TRANSCRIPTIONAL ANALYSIS OF HYDROGENASE GENES IN *RHODOBACTER SPHAEROIDES* O.U.001

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Approval of the Graduate School of Natural and Applied Sciences

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I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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Nihal DOĞRUSÖZ

ABSTRACT

TRANSCRIPTIONAL ANALYSIS OF HYDROGENASE GENES IN *RHODOBACTER SPHAEROIDES* O.U.001

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In photosynthetic non-sulphur bacteria, hydrogen production is catalyzed by nitrogenases and hydrogenases. Hydrogenases are metalloenzymes that are basically classified into: the *Fe* hydrogenases, the *Ni-Fe* hydrogenases and *metal-free* hydrogenases. Two distinct Ni-Fe hydrogenases are described as uptake hydrogenases and bidirectional hydrogenases. The uptake hydrogenases are membrane bound dimeric enzymes consisting of small (*hupS*) and large (*hupL*) subunits, and are involved in uptake and the recycling of hydrogen, providing energy for nitrogen fixation and other metabolic processes.

In this study the presence of the uptake hydrogenase genes was shown in *Rhodobacter sphaeroides* O.U.001 strain for the first time and *hupS* gene sequence was determined. The sequence shows 93% of homology with the uptake hydrogenase *hupS* of *R.sphaeroides* R.V.

There was no significant change in growth of the bacteria at different concentrations of metal ions (nickel, molybdenum and iron in growth media).

The effect of metal ions on hydrogen production of the organism was also studied. The maximum hydrogen gas production was achieved in 8.4μM of nickel and 0.1 mM of iron containing media.

The expression of uptake hydrogenase genes were examined by RT-PCR. Increasing the concentration of Ni^{++} up to 8.4μM increased the expression of uptake hydrogenase genes (*hupS*). At varied concentrations of Fe-citrate (0.01 mM-0.1 mM) expression of *hupS* was not detected until hydrogen production stopped. These results will be significant for the improvement strategies of *Rhodobacter sphaeroides* O.U.001 to increase hydrogen production efficiency.

In order to examine the presence of *hupL* genes, different primers were designed. However, the products could not be observed by PCR.

Keywords: *Rhodobacter sphaeroides* O.U.001; Biohydrogen; uptake hydrogenase; RT-PCR

ÖZ

RHODOBACTER SPHAEROIDES O.U.001 HİDROJENAZ GENLERİNDE EKSPRESYON ÇALIŞMALARI

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Fotosentetik kükürt oluşturmayan bakterilerde hidrojen üretimi Nitrojenaz ve Hidrojenaz enzimleri tarafından kataliz edilir. Hidrojenazlar metal içeren enzimler olup üç ana gruba ayrılırlar; Ni-Fe Hidrojenazlar, Fe Hidrojenazlar ve Metalsiz Hidrojenazlar. Ni-Fe Hidrojenazları ise alım (Uptake) ve geri dönüşümlü (Reversible) hidrojenazlar olmak üzere iki gruba ayrılırlar. Alım hidrojenazları zara bağlı olan dimerik enzimlerdir. Küçük altbirim (*hupS*) ve büyük altbirimden oluşurlar (*hupL*) ve azot fiksasyonuna ve diğer metabolik olaylara enerji sağlayarak hidrojen alımında ve döngüsünde rol alırlar.

Bu çalışmada, *Rhodobacter sphaeroides* O.U.001'deki alım hidrojenaz genlerinin varlığı, küçük alt birime yönelik (*hupS*) hazırlanmış primerler ve PCR kullanılarak araştırılmıştır. Yapılan DNA izolasyonu ve PCR sonucunda beklenen

ürün elde edilmiş ve bu ürün için sekans analizi yaptırılmıştır (İontek, İstanbul). Ürünün primer dizaynında sekansı kullanılan *R.sphaeroides* RV suşuyla %93 oranında homoloji gösterdiği bulunmuş ve *R. sphaeroides* O.U.001 de Hidrojenaz genlerinin varlığı gösterilmiştir. Polimeraz zincir reaksiyonları bittikten sonra % 1,5 'luk agaroz jel elektroforez sisteminde yürütülerek sonuçlar incelenmiştir.

Farklı metal iyon derişimleri içeren (nikel, molibdenyum ve demir) besiyerinde çoğaltılan bakterilerin üreme eğrilerinde önemli bir fark gözlenememiştir.

Metal iyonların bakterilerin hidrojen gazı üretimi üzerindeki etkisi de bu çalışmada incelenmiştir. En yüksek hidrojen üretimi 8,4 μM nikel ve 0,1 mM demir içeren besiyerlerinde gözlenmiştir.

Farklı fizyolojik koşulların (farklı Ni^{++} ve Fe^{++} derişimleri) hidrojenaz enzimleri kodlayan genlerin ifadeleri üzerindeki etkisini anlamaya yönelik çalışmalar RT-PCR yöntemiyle yapılmıştır. Nikel derişimini 8,4 μM'e artırmak *hupS* gen ekspresyonunu artırmıştır. Değişen demir derişimlerinde (0,01-0,1 mM) hidrojen üretimi sonlanana kadar *hupS* ekspresyonu gözlenmemiştir.

Anahtar kelimeler: *Rhodobacter sphaeroides* O.U.001; alım hidrojenazı; RT-PCR

to my family

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ABBREVIATIONS

Acetyl-CoA	Acetyl Coenzyme A
ADP	Adenosine di-Phosphate
ATP	Adenosine tri-Phosphate
CTAB	Cetyltrimethylammonium bromide
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium Bromide
hupL	Uptake Hydrogenase Large Subunit
hupS	Uptake Hydrogenase Small Subunit
ICM	Intracytoplasmic membrane system
M-MuLV-RT	Moloney Murine Leukemia Virus Reverse Transcriptase
MOPS	3-(N-Morpholino)propanesulsonic acid
NCBI	National Center of Biotechnology Information
R.capsulatus	*Rhodobacter capsulatus*
R.sphaeroides	*Rhodobacter sphaeroides*
RLT	Rneasy Lysis Buffer
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
TCA	Tricarboxylicacid

CHAPTER 1

INTRODUCTION

1.1 Hydrogen

Hydrogen is the most abundant and the smallest element present in the universe. It constitutes almost 90 % of the universe by weight. Hydrogen is found in water that covers 70% of the earth's surface and in all organic matter. However, it is not commonly found in its pure form, since it readily combines with other elements such as oxygen and carbon (www.eere.energy.gov/hydrogenandfuelcells).

Hydrogen is a colorless, odorless, and nonpoisonous gas under normal conditions. The amount of energy produced by hydrogen, per unit weight of fuel, is about three times the energy contained in an equal weight of gasoline and nearly seven times that of coal (www.fsec.ucf.edu/hydrogen).

1.1.1 Hydrogen As a Fuel

Hydrogen presents an exciting option as the alternative fuel of the future. As a nearly ideal energy carrier, hydrogen will play a critical role in a new, decentralized energy infrastructure that can provide power to vehicles, homes, and industries.

Conventional petroleum-based fuels like gasoline, diesel, as well as natural gas and coal, all contain carbon. When these fuels are burned, their carbon

recombines with oxygen from the air to form carbon dioxide (CO_2), the primary greenhouse gas that causes global warming.

Furthermore, combustion of fossil fuels at the high temperatures and pressures reached inside an internal combustion engine (what powers most vehicles) or in an electric power plant produces other toxic emissions. Carbon monoxide (a poison), oxides of nitrogen and sulfur, volatile organic chemicals, and fine particulates are all components of air pollution attributable to the refining and combustion of fossil fuels. When released into the atmosphere, many of these compounds cause acid rain or react with sunlight to create ground-level smog. Vast ecosystem damage, increased lung disease and cancer are the ultimate price we pay for consuming these fossil fuels. (http://www.rmi.org)

It is also important to consider that fossil fuels are finite. Current energy systems are inadequate in terms of their ability to meet increasing demand for the future.

Hydrogen can be totally nonpolluting (water is the exhaust), economically competitive, as safe as gasoline, diesel, or natural gas, and help prevent the depletion of fossil fuel reserves.

1.1.2 Use of Hydrogen

Hydrogen is currently used in many different industry processes, such as the production of plastics, fertilizers and petroleum products. It can also be used for hydrogenation of fats and oils, in which vegetable oils are changed to solid form.

Hydrogen may be used to power steam turbines or as fuel in a vehicle internal combustion engine. It can also be used in fuel cells to power a wide variety of applications, both mobile and stationary, small- and large-scale. Fuel cells can be used to provide clean energy for transportation. And because they are modular, fuel cells can provide heat and electricity for homes or to supply the energy to run an entire large commercial building.

(http://www.eere.energy.gov/hydrogenandfuelcells/hydrogen/potential.html)

1.2 Production of Hydrogen

Most methods of producing hydrogen involve splitting of water into its components hydrogen and oxygen. The most of these methods are given below:

- Steam reforming converts methane (and other hydrocarbons in natural gas) into hydrogen and carbon monoxide by reaction with steam over a nickel catalyst
- Electrolysis uses electrical current to split water into hydrogen at the cathode (+) and oxygen at the anode (-)
- Steam electrolysis (a variation on conventional electrolysis) uses heat, instead of electricity, to provide some of the energy needed to split water, making the process more energy efficient
- Thermochemical water splitting uses chemicals and heat in multiple steps to split water into its component parts
- Photoelectrochemical systems use semi-conducting materials (like photovoltaics) to split water using only sunlight

- Thermal water splitting uses a very high temperature (approximately 1000°C) to split water
- Gasification uses heat to break down biomass or coal into a gas from which pure hydrogen can be generated
- Photobiological systems use microorganisms to split water using sunlight
- Biological systems use microorganisms to break down a variety of biomass feedstocks into hydrogen. (http://www.hydrogenus.com/h2-production.asp)

1.2.1 Photobiological Hydrogen Production

Biological production of hydrogen, using microorganisms, is an exciting new area of technology development that offers the potential production of usable hydrogen from a variety of renewable resources. In Figure 1.1, the concept of biological hydrogen production and its utilization was shown. Biological systems provide a wide variety of approaches to generate hydrogen include biophotolysis of water using algae and cyanobacteria, photodecomposition of organic compounds by photosynthetic bacteria, fermentative hydrogen production from organic compounds and hybrid systems using photosynthetic and fermentative bacteria (Das *et al*., 2001).

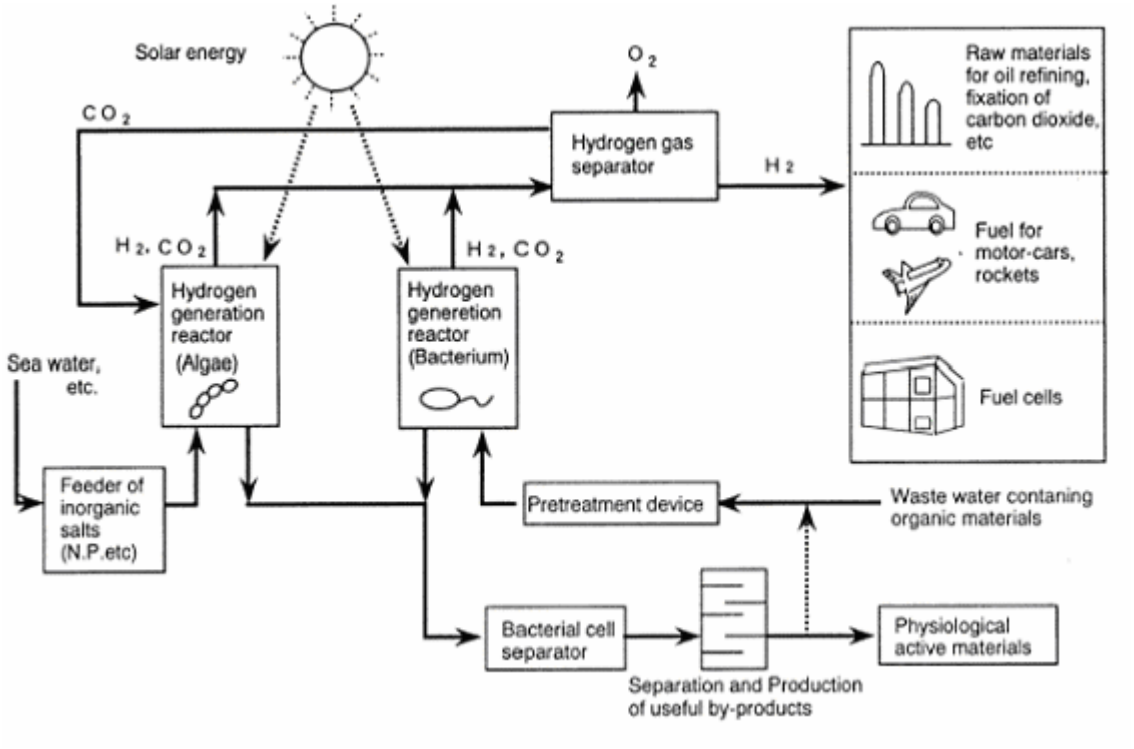

Figure 1.1 Concept of biological hydrogen production and utilization. (Miyake et al., 1999)

Green algae and cyanobacteria directly decompose water to hydrogen and oxygen with light energy:

$$H_2O \rightarrow H_2 + 1/2O_2$$

The reaction requires only water and sunlight, however, the process shows rather low efficiency of hydrogen production due to the complicated reaction system which remains to overcome the large free energy (+242 kJ mol^{-1}).

Photosynthetic bacteria do not utilize water as the starting material for the production of hydrogen but, rather, use organic acids.

$$\{CH_2O\}_x + xH_2O \rightarrow 2xH_2 + xCO_2$$

When compared to the algal hydrolysis, the process requires less light energy to produce hydrogen. There are many kinds of photosynthetic bacteria and organic

substrates (Miyake *et al*., 1999). Organic acids, sugars, sulfur compounds, food wastes, agricultural wastes, organic waste waters can be used as starting materials of photobiological production of hydrogen (Wakayama *et al*., BiohydrogenII).

Photosynthetic bacteria are indicated as the most promising microbial system for the biological hydrogen production. The major advantages are:

- high theoretical conversion yields
- lack of O_2-evolving activity (O_2 causes inactivation problem in different biological systems)
- ability to use wide spectrum of light
- ability to consume organic wastes and potential usage in association with wastewater treatment (Das *et al*., 2001)

1.3 The Photosynthetic Bacteria

The photosynthetic bacteria are aquatic Gram-negative organisms found in a wide range of environments, including marine and freshwater systems. Because they can utilize solar energy for the fixation of CO_2 and N_2, they represent an important component of these ecosystems (Pfenning and Truper, 1974; Truper and Pfenning, 1981). Unlike cyanobacteria, they contain only one photosystem and perform anoxygenic photosynthesis (van Niel, 1931; Clayton and Sistrom, 1978).

Photosynthetic bacteria are also known as green and purple bacteria or Anoxyphotobacteriae (Gibbons and Murray, 1978) do not produce oxygen. They contain two orders based on differences in the fine structure and pigment content of the photosynthetic apparatus, the Rhodospirillales (“purple bacteria”) and the

Chlorobiales (Truper and Pfenning, 1981). Photosynthetic bacteria have been classified into four families based on the physiological and ecological observations. The order Rhodospirillales comprises two families: the Rhodospirillaceae, purple non-sulphur bacteria (Pfenning and Truper, 1974) and the Choromatiaceae, purple sulphur bacteria (Bavendamm, 1924).

Photosynthetic non-sulphur bacteria family includes Rhodospirillum, Rhodocyclus, Rhodopseudomonas, Rhodomicrobium and Rhodobacter. Production of hydrogen studies is mostly concentrated on Rhodobacter genera.

1.3.1 *Rhodobacter sphaeroides* O.U.001

R.sphaeroides O.U.001 is one of the Purple Non-Sulphur Bacteria placed under the Rhodobacter genus along with *R.capsulatus*.

This group of bacteria are among the most metabolically diverse organisms known, being capable of growing in a wide variety of growth conditions. They can grow as photoheterotrophs under anaerobic conditions in the light with many different organic compounds as carbon and electron sources. They can also grow well under aerobic dark conditions at the full oxygen tension of air. Anaerobic dark fermentative metabolism with pyruvate and sugars allows only mariginal growth (van Niel, 1944). *R.sphaeroides* employ tight control and regulation of these alternative metabolisms and are able to shift from one mode of metabolism to another, in order to attain the optimum metabolic efficiency with respect to varying conditions.

Anaerobically grown cultures of *R.sphaeroides* O.U.001 are dirty greenish brown to dark brown in color. When the culture is exposed to air the color will change into red gradually (Holt *et al*., 1984).

Cells of *R.sphaeroides* O.U.001 vary considerably in morphology especially when grown in complex media. Cells are ovoid, heart shaped or short rods in defined media. Many cells occur in pairs, sometimes connected by a slender filament or tube (Pellerin and Gest, 1983).

Although they are called Purple Non-Sulphur Bacteria, *R.sphaeroides* O.U.001 can use sulphide if presents in low concentrations. Higher concentrations of H_2S are toxic (Lindquist, 1999). They can be found in ponds, lakes and lagoons. Because an active breakdown of organic matter takes place in those places, vertical gradients of oxygen and light(from above) and hydrogen sulphide (from below) are constituted (Caumette, 1984). Therefore bacterial gradient in these aquatic ecosystems can be seen. *R.sphaeroides* O.U.001 are found in upper, more aerated waters with low sulphide content (Caumette *et al*., 1983).

1.3.2 Hydrogen Production and Utilization by *R.sphaeroides* O.U.001

Production of hydrogen occurs from the breakdown of organic substrates in the presence of an inert, anaerobic atmosphere such as argon under illumination in vitro conditions. The cells obtain carbon from organic substrates and energy from light. Nitrogen should be limited and ammonia should be absent from the culture medium for the hydrogen production.

The reaction mechanism of hydrogen production can be shown in Figure 1.2. The hydrogen production system involves three important components; the TCA

cycle, the photosynthetic membrane apparatus and the enzyme complex. The carbon substrate (intermediate of the TCA cycle) is fed into the TCA cycle where it is oxidized to produce CO_2 and electrons. Light energy is converted to the potential energy of the electron, which then forms ATP by photosynthetic membrane apparatus. In another process, the elevated electron is needed to reduce ferrodoxin (Fd), a typical electron carrier to the hydrogen-producing enzyme, nitrogenase. ATPs produced are supplied to the enzyme together with the electron carried. Photons activate the photosystem in the reaction center to pump protons. Proton transport couples with the generation of ATPs. It has not been well elucidated how many photons are required to give a mole of ATP (Oasawa *et al*., 1984; Miyake *et al*., 1999).

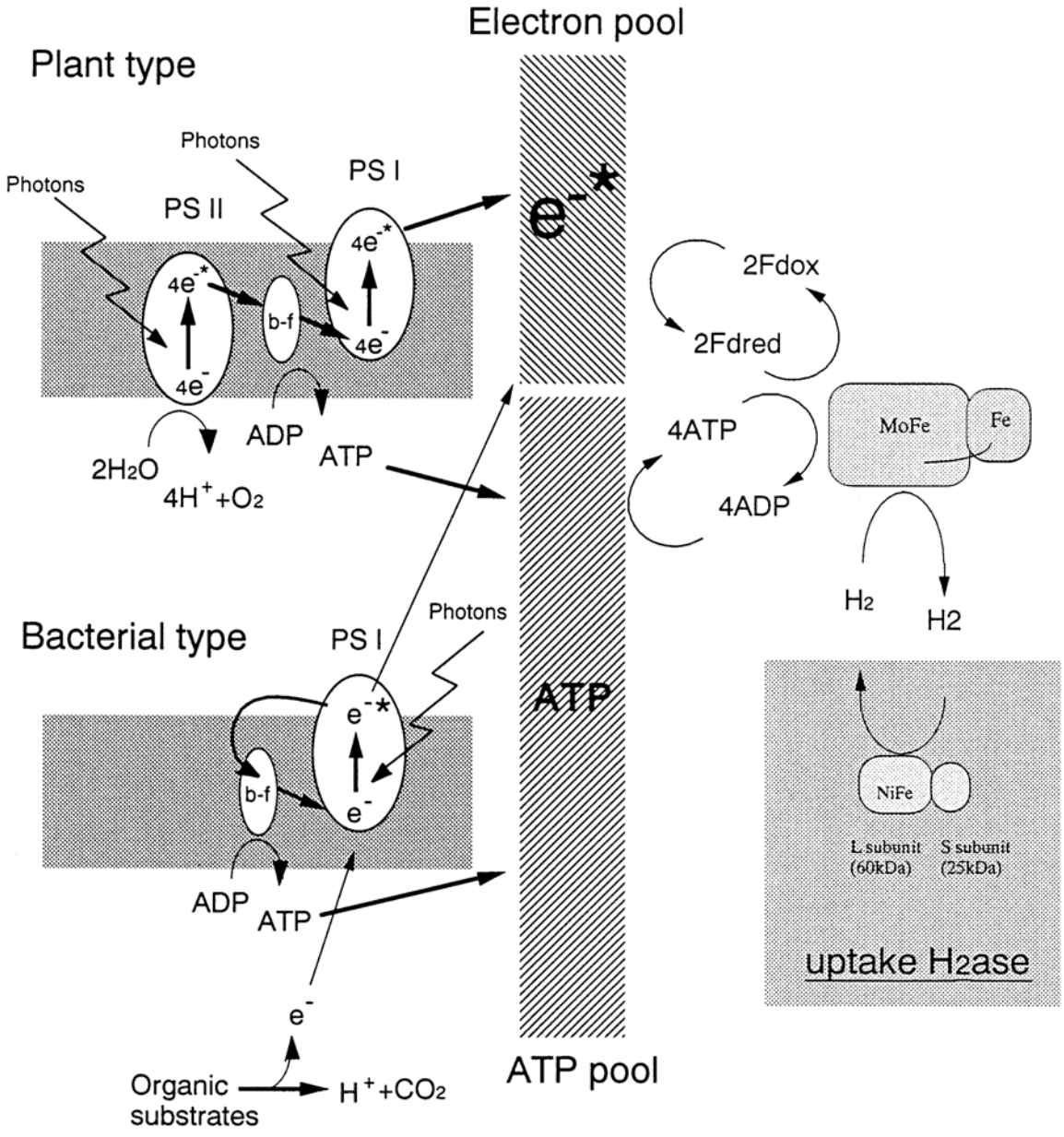

Figure 1.2 Mechanism of light to hydrogen conversion by photosynthetic bacteria (Miyake *et al*., 1999)

1.4 Enzymes Involved in Hydrogen Production

Hydrogen production in Photosynthetic Non-sulphur Bacteria requires two enzyme systems: Nitrogenases and Hydrogenases.

1.4.1 Nitrogenase

In all known biological systems, nitrogen fixation that is essential for the maintenance of the nitrogen cycle is catalyzed by the enzyme nitrogenase

$$N_2+8H^++8e^-+16MgATP\rightarrow 2NH_3+16MgADP+16Pi+H_2$$

Nitrogen fixation to ammonia catalyzed by nitrogenase complex is a highly endergonic reaction requiring ATP. The physiological substrate of nitrogenase is molecular nitrogen. Moreover, this reaction is accompanied by an obligatory reduction of protons ($8H^+$) to molecular hydrogen.

When the physiological substrate absent, nitrogenase catalyzes the reduction of protons and hydrogen is the primary product:

$$2\ H^++2e^-+4ATP\rightarrow H_2+4ADP+4Pi$$

Nitrogenase complex was first isolated from an anaerobic non-photosynthetic bacterium, *Azobacter vinelandii* (Bulen and Lecomte, 1966). There are three genetically distinct nitrogenase systems (*nif, vnf, anf*) exist in nature (Schneider *et al*., 1997). The classical Mo-containing nitrogenase (*nif* system) is the most widespread and intensively characterized system. Two types of alternative Mo-independent nitrogenases have been identified. Vanadium containing nitrogenase system (vnf system) was first reported by Eady *et al*. (1987) and then characterized (Eady, 1991). The other alternative Mo-independent nitrogenase system is Fe

nitrogenase lacking both vanadium and vanadium (*anf* system) (Muller *et al*., 1992, Krahn *et al*., 1996).

The nitrogenase complex consists of two proteins: the dinitrogenase (MoFe protein or protein I) and the dinitrogenase reductase (Fe protein or protein II), (Figure 1.3) (Vignais *et al*., 1985, Burris, 1991, Howard and Rees, 1994).

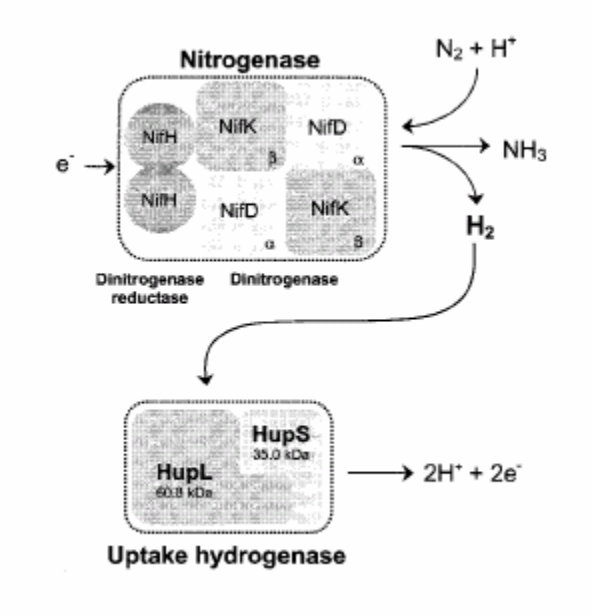

Figure 1.3 Enzymes involved in hydrogen production (Tamagnini *et al*., 2002)

The mechanism of nitrogenase is divided into two parts; the redox cycle between the Fe protein and the MoFe-protein, and the substrate reduction cycle (Howard and Rees, 1994). The electron transfer proceeds from the Fe-protein (4Fe-4S) to the MoFe-protein and 2 ATP hydrolysis occur per electron transferred. Only the complex of Fe-protein/MoFe-protein turns over ATP, not Fe-protein-MgATP (ATP and ADP are bound by the Fe-protein). The substrate reduction rate is dependent on both the ratio of the two protein components and the absolute protein

concentrations and there is a burst of ATP hydrolysis before substrate reduction activity (Figure 1.4).

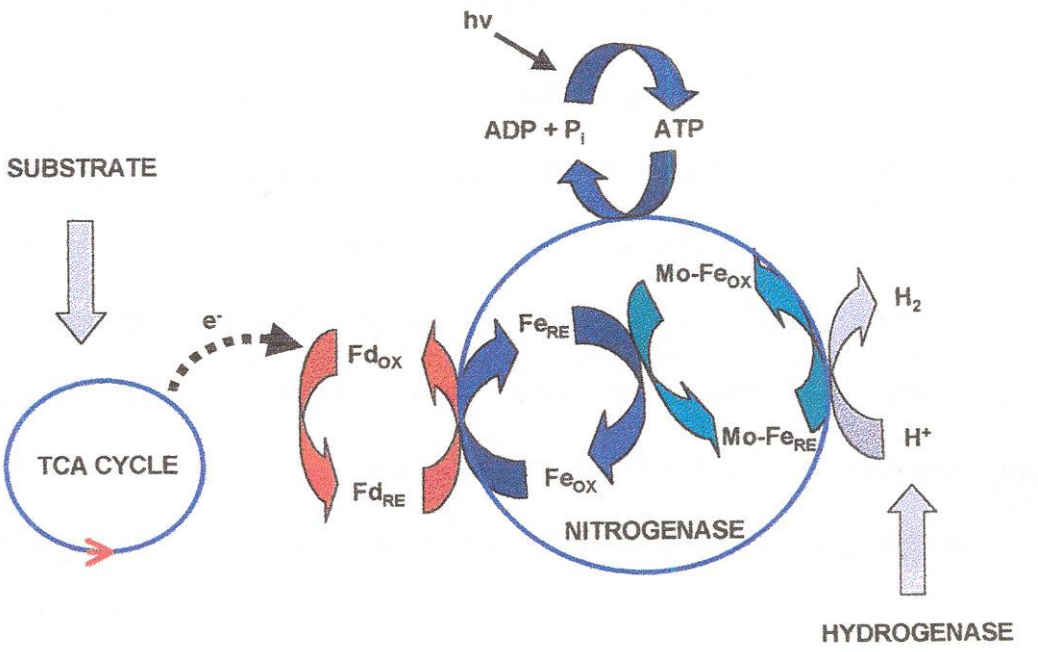

Figure 1.4 Mechanism of Nitrogenase and Functional Analysis of TCA Cycle and Hydrogenase (Burges, 1984)

Nitrogenase is very oxygen labile. Because it permanently deactivates and represses the synthesis of the enzyme, organisms must have mechanisms for the protection. Nitrogenase complex of photosynthetic bacteria can function in the presence of oxygen at concentrations lower than air (Roberts and Brill, 1981).

Ammonia or ammonium salts used as fixed nitrogen sources are also inhibitors of nitrogenase complex in *R.sphaeroides* (Jones and Monthy, 1979). Glutamine and other fixed nitrogen sources are known to provoke a similar effect. After removal of excess fixed nitrogen by washing or consumption by the cells, the

activity of the enzyme is restored completely. This regulatory process is called "switch-off" effect (Zumft and Castillo, 1978 as cited in Vignais *et al*., 1985).

Another factor regulatory for the nitrogenase complex is molecular nitrogen (N_2). In the presence of molecular nitrogen, nitrogen fixation reaction is favored.

Light availability is also important for the hydrogen production activity of nıtrogenase complex. Nitrogenase synthesis can occur in the dark, at non-inhibitory oxygen tensions. Nevertheless, it was found that light strongly stimulates nitrogenase activity of whole cells. Synthesis of the enzyme is related to bacterial photophosphorylation activity (Reidl *et al*., 1983). The photophosphorylation capacity is greater in cells grown under high light intensity than in cells grown under low light intensities.

1.4.2 Hydrogenase

Hydrogenases are enzymes that reversibly catalyze the production or consumption of hydrogen and play a central role in microbial energy metabolism: $H2 \leftrightarrow 2H+2e^-$. This reaction is coupled with the reduction or oxidation of an associated electron carrier specifically interacting with the enzyme (Tosi *et al*., 1998). Its presence enables microorganisms to either use H_2 as a source of energy and reductant, or use protons as a terminal electron acceptor.

These enzymes have been purified from a wide variety of microorganisms and extensively characterized with regard to structure, catalytic properties and genetic organization (Adams, 1990; Reeve and Beckler, 1990; Przybyla *et al*., 1992; Wu and Mandrand, 1993; Friedrich and Schwartz, 1993; Albracht, 1994; Vignais and

Toussaint, 1994). The enzyme as capable of using H_2 as a substrate was first demonstrated by Stephenson and Stickland (1931) who named it "hydrogenase".

Various microorganisms can use H_2 as an electron source either aerobically or anaerobically (Meyer *et al*., 1978; Vignais *et al*., 1985). These various functions are often associated with different cellular localizations. Photosynthetic bacteria hydrogenases are generally membrane bound, however the enzyme may be bound to the membrane more or less (Vignais *et al*., 1989). Since the protons resulting from the oxidation of H_2 are discharged in the cytoplasmic compartment, it follows that enzyme is a transmembrane protein (Doussiere *et al*., 1980).

The hydrogenases of photosynthetic bacteria show high resistance to oxygen unlike nitrogenases. They remain intact and active even inactivated reversibly in high concentrations of oxygen (Vignais *et al*., 1985).

Hydrogenases are resistant to temperatures up to about 80 ˚C. In membrane bound enzyme, the inactivation rate is greater. This result may be because of loosing ability to change conformation into a more stable form.

Photosynthetic bacteria hydrogenases have a high resistance to denaturing agents. High concentrations of detergents are required for extraction from membrane and solubilization (Kovacs and Bagyinka, 1990).

Hydrogenases that have been characterized are iron-sulfur proteins that contain Fe atoms arranged in Fe-S clusters. They can be distributed into three major groups according to their metal compositions; the [Fe]-hydrogenases, the [NiFe]-hydrogenases and the metal-free hydrogenases (Vignais *et al*., 2001). Spectroscopic data indicates that the Ni metallocenter is directly involved in H_2 activation

(Albracht, 1994). The majority of hydrogenases are [NiFe]-hydrogenases found in all bacterial classes. Therefore, they have been the most extensively studied.

1.4.2.1 [NiFe]-hydrogenases

[NiFe]-hydrogenases isolated from different kinds of bacteria are very similar in their subunit composition. Two distinct [Ni-Fe] hydrogenases have been described as an uptake enzyme and a bidirectional enzyme. The uptake hydrogenase with the function of catalyzing the consumption of the hydrogen produced by nitrogenase is a dimeric enzyme consisting of a large subunit (*hupL*) and a small subunit (*hupS*) (Kovacs and Bagyinka, 1990). Large subunit carries the nickel containing active site and its molecular mass ranges from 45 kDa to 65 kDa. Small subunit is approximately 28-35 kDa and it includes Fe-S clusters (Tosi *et al*., 1998).

The bimetallic NiFe center of the active site is located in the large subunit and is deeply buried inside the protein. The small subunit contains up to three Fe-S clusters, which conduct electrons between the H_2 activating center and the physiological electron acceptor or donor of hydrogenases (Figure 1.5 and Figure 1.6) (Vignais *et al*., 2001).

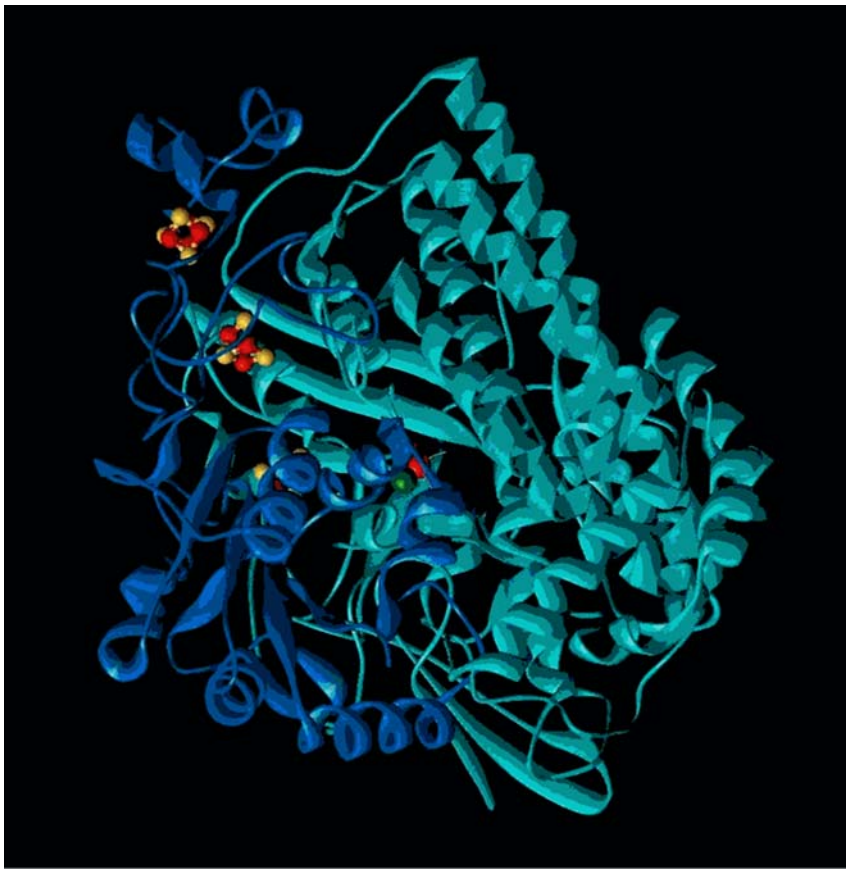

Figure 1.5 Three dimentional structure of *Desulfovibrio gigas* Hydrogenase (http://www.chem.ox.ac.uk/icl/faagroup/hydrogenase.html)

[NiFe]-hydrogenases have been grouped into four subclasses according to the evolutionary relationships and motif compositions or the different enzymes (Wu and Mandrand, 1993);

- membrane-associated respiratory uptake [NiFe]-hydrogenases (Group1)
- cytoplasmic heterodimeric [NiFe]-hydrogenases (Group 2)
- cytoplasmic heteromultimeric reversible [NiFe]-hydrogenases (Group 3)

- the membrane-associated H_2-evolving respiratory [NiFe]-hydrogenases (Group 4)

R.sphaeroides hydrogenases belong to the group 1 [NiFe]-hydrogenases. These so-called respiratory uptake hydrogenases are enzymes capable of supporting growth with H_2 as an energy source. The respiratory hydrogenases of Group 1 transfer electrons from H_2 to a cytochrome that is bound to a membrane-located complex coupling electron transfer to trans-membrane proton translocation (Vignais *et al*., 2001).

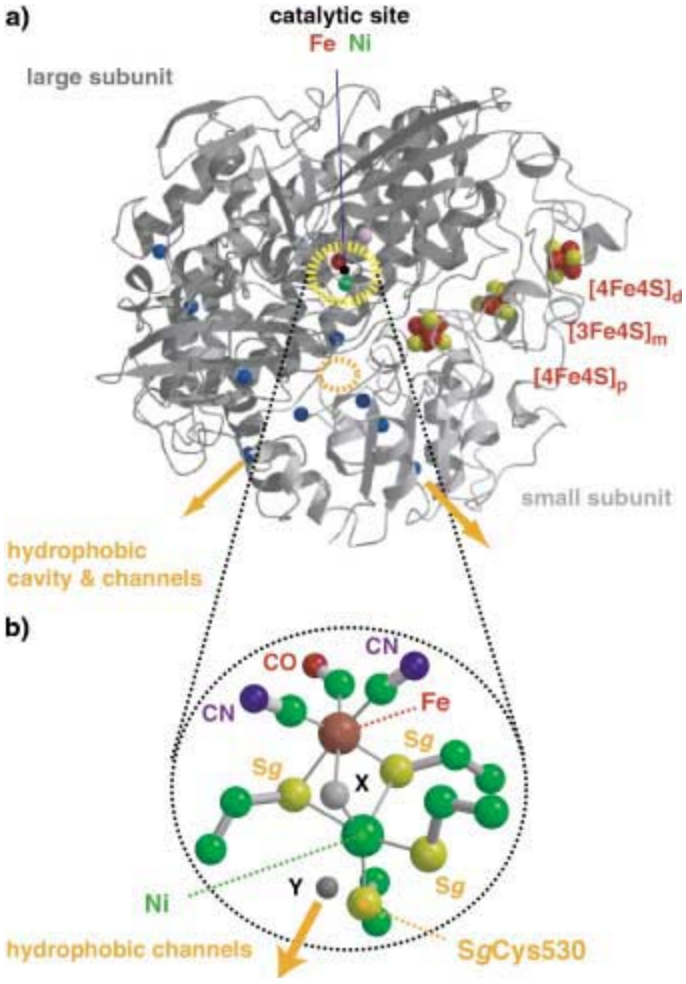

Figure 1.6 The overall structure of NiFe hydrogenases
a) Structure of *D.gigas* enzyme. The polypeptide folds of the large and small subunits are colored in dark and light grey respectively. The catalytic center NiFe and iron-sulfur cluster atoms are represented by spheres.
b) Close view of the catalyatic site.
(Frey, M., 2002)

1.4.2.1.1 Uptake Hydrogenase

As the organism has no way to eliminate H_2 production, their only way is to recycle the H_2 to recover some of its energy. With hydrogenase, they can oxidize H_2 and couple this reaction to ATP formation by generating membrane potential (Meyer *et al*., 1978). In this process, hydrogenase transfer electrons from H_2 to the membrane bound electron chain. The primary electron acceptor is probably either a ubiquinone or cytochrome b. Hydrogenases plays a significant role in the membrane bioenergetic process by coupling oxidation of H_2 to ATP synthesis through generation of membrane potential (Kovacs and Bagyinka, 1990).

There are some evidences that H_2 produced by nitrogenase is recycled by hydrogenase. H_2 recycling and nitrogenase activities are inhibited by inhibitors of low potential electron carriers such as ferrodoxin (Vignais *et al*., 1985).

1.4.2.2 Organization of Hydrogenase Genes

The small and large subunits of hydrogenase are encoded by the *hupS* and the *hupL* structural genes. Proteins of these genes share a high degree of identity of the [NiFe]-hydrogenases belonging to group 1 in the classification of Wu and Mandrand (1993) (Colbeau *et al*., 1993).

The *hupS* gene is preceded by a sequence encoding a signal peptide of 45 amino acids, which is found not only in periplasmic hydrogenase encoding genes but also is highly conserved among the membrane bound hydrogenases (Vignais and Toussiant, 1993). “RRXFXK” consensus element is found in all putative cleavable signal peptides at the N-terminal end of small subunit (Voordouw, 1992). Those

signal peptides may function for membrane integration and for protein export to the periplasm (Colbeau *et al*., 1983).

Up to 20 accessory genes were found to be linked to *hupS* and *hupL,* whose products are not a constitutive part of the mature enzyme, but rather function as potential electron carriers channeling electrons from the enzyme to the natural electron acceptor, as positive or negative regulators, as proteins taking part in nickel metabolism, and as proteins involved in hydrogenase activation steps (Tosi *et al*., 1998). A set of proteins, encoded by the *hyp* genes is involved in the insertion of Ni, Fe, CO and CN into the active site (Vignais *et al*., 2001).

1.4.2.3 Regulation of Hydrogenase Genes

Metabolic versatility of the Purple Non-Sulphur bacteria permits their adaptability of environmental changes. Hydrogenase gene expression in response to environmental stimuli has been studied only in *Rhodobacter capsulatus*. There are two factors affecting hydrogenase gene expression with respect to environmental stimuli; the availability of molecular hydrogen and the levels of O_2 in the environment. Synthesis of hydrogenase is stimulated by H_2 and is repressed by O_2. However, expression is maximal in the presence of H_2 and O_2 (Vignais *et al*., 1998).

1.5 Overall Hydrogen Production Mechanism

There are three factors affecting hydrogen production in Purple Non- Sulphur Bacteria under illumination in anaerobic conditions; TCA cycle, photosynthetic membrane apparatus and enzyme complex (Figure 1.7; Koku *et al*., 2002).

Photosynthetic membrane apparatus converts light energy into ATP which is directed to nitrogenase along with protons and electrons. Protons are supplied by the TCA cycle in partly and ATP-synthase supplies the remaining working as a part of photosynthetic apparatus. Finally, nitrogenase reduces protons to molecular hydrogen. Hydrogenase recycles the produced hydrogen. Therefore, the net hydrogen amount produced is that the difference between the amount nitrogenase produced and the amount hydrogenase consumed.

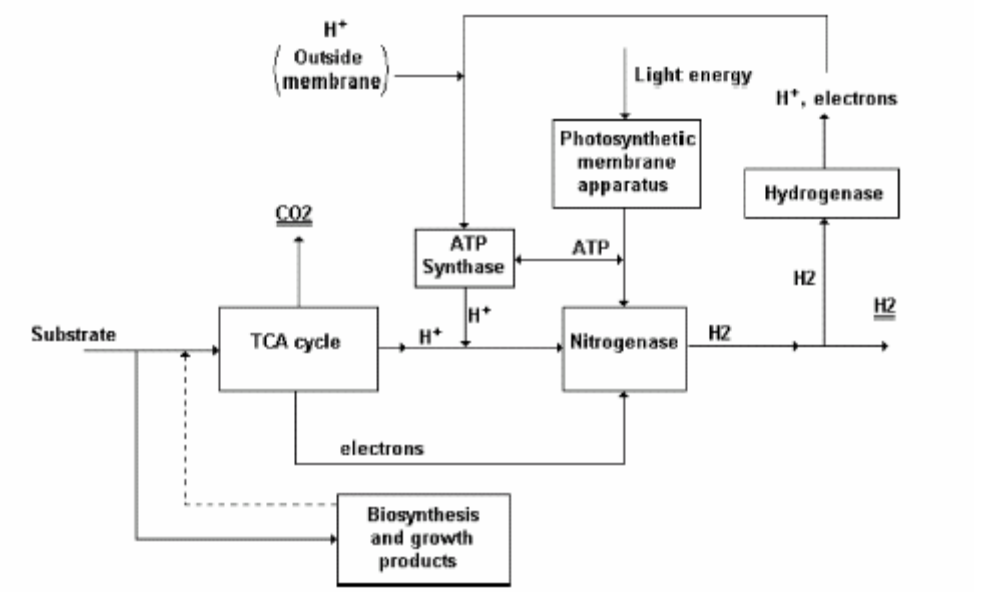

Figure 1.7 The overall scheme of hydrogen production by PNS bacteria (Koku *et al*., 2002)

1.6 Objectives of This Study

The main objective of this study was to show the presence of uptake hydrogenase (*hupSL*) in the *R. sphaeroides* O.U.001 and to examine transcriptional regulation of hydrogenase genes under different physiological conditions. For this purpose primers were designed for *hupS* gene and expression was studied by Reverse

Transcriptase-Polymerase Chain Reactions (RT-PCR). The effect of various concentrations of metal ions (Nickel, Iron and Molybdenum) on growth of bacteria and hydrogen production was also investigated.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Strain

R. sphaeroides O.U.001 (DSM 5864) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany as freeze-dried culture. The bacteria were activated by rehydration of dried cultures with a sterile liquid rich medium. Activated cultures were transferred continuously into new growth medium (Biebl and Pfenning, 1981) to keep them active.

2.2 Reactivation Media and Culture Media

The composition and the methods of preparation for reactivation and for culturing are described in Appendix A.

2.3 Solutions and Buffers

Composition of solutions and buffers are given in Appendix A.

2.4 The chemicals and Enzymes

The chemicals and enzymes and their suppliers are listed in Appendix A.

2.5 Growth Conditions and Maintenance of Bacterial Strains

Anaerobic photoheterotrophic growth mode is the optimum for *R. sphaeroides* O.U.001, with malic acid as the carbon source, sodium glutamate as the nitrogen source, vitamin and trace element solution under illumination, among

different modes of growth such as photoautotrophy, chemoautotrophy, fermentative, photoheterotrophy and chemoautotrophy.

In this study, the bacteria was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany as freeze-dried culture, it was activated by rehydration of dried cultures with a sterile liquid rich medium and the culture was transferred continuously into new growth medium to keep them active.

2.5.1 Anaerobic Cultivation Liquid Media

After the activation with a modified Pfenning's Rich Medium (1988), bacteria were maintained in the Minimal Medium of Biebl and Pfenning (1981) modified by omitting ammonium chloride and yeast extract. 7.5 mM malic acid was used as the carbon source and 10 mM sodium glutamate was used as the nitrogen source.

Anaerobic cultivation of *R. sphaeroides* O.U.001 was performed in 25 or 50 ml penicillin bottles with rubber caps. After adjustment of pH 6.8, prepared medium was distributed into the bottles and sterilized by an autoclave for 15 min. at 121 ˚C. Pure argon gas (99.995 % purity) was flushed into the bottles with a rate of 100 ml/min to create an anaerobic atmosphere using sterile syringes and 0.20-micron syringe filters. Finally, 10% inoculation of bacteria into prepared anaerobic liquid media was done. The bottles were incubated at 30 ˚C illuminated by 100 W tungsten lamps at 10-15 cm distance.

For the long term storage of bacteria, sterile glycerol which prevents freezing was used. Cultures grown in anaerobic liquid media at 30 ˚C under illumination until

mid-log phase was inoculated into the anaerobic liquid media containing 10% of glycerol, the contents were mixed and the bottles were stored at -80 ˚C.

2.6 Growth Mediums Containing Different Concentrations of Nickel, Iron and Molybdenum

Sets of growth mediums containing different Nickel [$NiCl_2.6H_2O$(0.084µM)], Molybdenum [$Na_2MoO4.2H_2O$(0.165µM)], and Iron [Fe(III)citrate.hydrate(0.1mM)] concentrations (1/10, 10 and 100 folds) were shown in Table 2.1.(Appendix A).

2.7 Growth of *R.sphaeroides* O.U.001

Modified Biebl and Pfenning Minimal Medium in 100 ml penicillin bottles was used (Appendix A) for the construction of growth curves. 10 different sets of medium containing different nickel, molybdenum and iron concentrations which are given in Table 2.1 were used.

Table 2.1 Mediums containing different concentrations of nickel, iron and molybdenum. For instance, "6 (Fe 10X)" means medium number 6 has 10 times as much iron as the control medium.

	[$NiCl_2.6H_2O$]	**[$Na_2MoO4.2H_2O$]**	**[Fe(III)citrate.hydrate]**
1 (Control)	0.084µM	0.165µM	0.1mM
2 (Ni 1/10X)	**0.0084µM**	0.165µM	0.1mM
3 (Ni 10X)	**0.84µM**	0.165µM	0.1mM
4 (Ni 100X)	**8.4µM**	0.165µM	0.1mM
5 (Fe 1/10X)	0.084µM	0.165µM	**0.01mM**
6 (Fe 10X)	0.084µM	0.165µM	**1mM**
7 (Fe 100X)	0.084µM	0.165µM	**10mM**
8 (Mo 1/10X)	0.084µM	**0.0165µM**	0.1mM
9 (Mo 10X)	0.084µM	**1.65µM**	0.1mM
10 (Mo 100X)	0.084µM	**16.5µM**	0.1mM

Active culture (10%) was inoculated into 70 ml anaerobic, sterile, liquid growth medium containing 100 ml penicillin bottles by sterile syringe. The experiment was started by placing the bottles into the incubator at 30 ˚C. Illumination was provided by a 100 W tungsten lamp at the distance 10-15 cm from the bottles. The time of inoculation was considered as zero-time and samples were taken from the bottles at 4-6 hours intervals while flushing with pure argon gas. The optical density of the samples which provides bacterial cell concentration determinations were measured at 660 nm by a spectrophotometer (Shimadzu UV-1201). Fresh medium was used as a blank solution.

2.8 Dry Cell Weight Analysis

For the determination of the bacterial dry cell weight, 1-1.5 ml of samples was taken from the cultures that were inoculated 10% at every 4-6 hours. Samples were centrifuged at 13,000 g for 10 min in microfuge and the pellets were dried overnight at 40 ˚C in an oven. Then, the dried pellets were weighed.

Dry cell weight versus OD_{660} was obtained from the samples corresponding to the various points of the growth curve (Appendix C).

2.9 Hydrogen Production

Production of hydrogen was studied using 55 ml penicillin bottles under illumination of 100 W tungsten lamp at 10-15 cm distance. Bieble and Pfenning Medium containing malate/glutamate (15 mM/2 mM) was used during hydrogen production experiments (Eroglu *et al*., 1999). Experimental setup is shown in Figure

2.1 and Figure 2.2. Two sets of cultures were prepared under the same conditions. Penicillin bottles completely filled with medium were inoculated by overnight culture to create an anaerobic atmosphere. First set of penicillin bottles filled with culture was connected to tubes including water with small diameter waterspouts. There were needles at the bottoms of tubes. When there is hydrogen gas production, it passed to tubes through waterspouts and water released out of tubes. Hydrogen gas produced by the culture was collected and measured by examining the amount of water replaced with hydrogen gas through tubes (Uyar *et al*., 2003). As the samples were taken for the determination of growth, pH and hydrogen gas production, about 1.5 ml of second set of culture was replaced with the culture in the first set. By this way conditions for hydrogen gas production such as anaerobic atmosphere was kept stable.

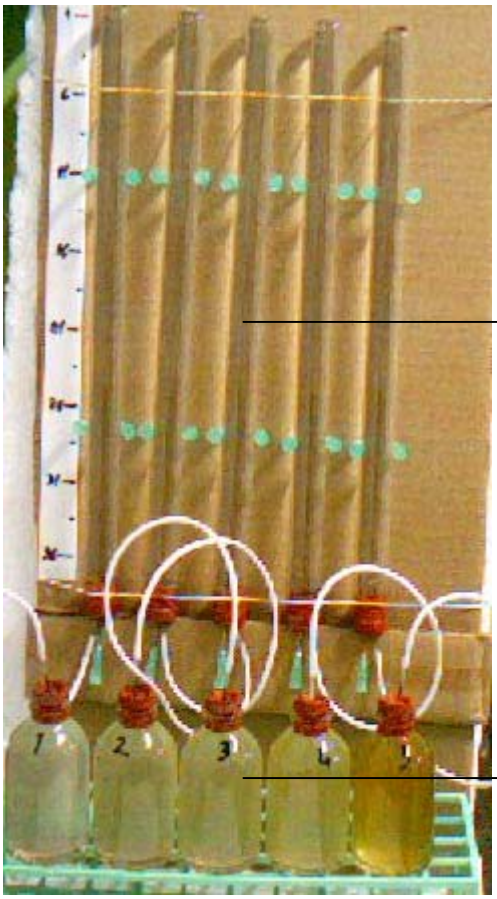

Figure 2.1 Experimental set up for hydrogen growth

Figure 2.2 Experimental set up for hydrogen production side-view

2.10 Genomic DNA Isolation

The procedure of M.Bazzicalupo and S.Fancelli was used for isolation of genomic DNA from *R. sphaeroides* O.U.001. The organism was grown up to mid-log phase. 1.5 ml of the culture was collected by centrifugation for 2 min at 8,000 rpm. The pellet was suspended in 500 µl of TE (Appendix A) and 30 µl of SDS together with 5 µl of Proteinase K were added. The tube was mixed by inversion and incubated at 37 ˚C for 1 h to allow cell lysis. After addition of 100 µl of 5 M NaCl, it was vortexed for few seconds. Then, 80 µl of CTAB (Cetyltrimethylammonium bromide) was added and the mixture was heated for 10 min at 65 ˚C. An equal volume (about 800 µl) of chloroform: isoamyl alcohol (24:1) was added, the tube

was vortexed for few seconds, then centrifuged 5 min at 11,000 rpm. The aqueous upper phase was collected in a new tube and equal volume of phenol: chloroform: isoamyl alcohol was added. After vortexing the mixture, centrifugation was done 5 min at 11,000 rpm. The upper aqueous phase was recovered in a fresh tube, 2 µl of RNAse was added and incubated 30 min at 37 ˚C. Approximately an equal volume of isopropanol was added, DNA was precipitated for 5 min at room temperature and centrifuged for 5 min at 11,000 rpm. The supernatant was discarded, the pellet was washed with 70% ethanol and then centrifuged 5 min at 11,000 rpm. Finally, the pellet was dried and solubilized in 10-20 µl of sterile water.

2.10.1 Spectrophotometric Analysis of DNA

10 µl of DNA isolate was diluted with 990 µl of TE buffer (pH 8.0) (Appendix A) in a quartz cuvette and the absorbances at 260 nm and 280 nm were measured (Shimadzu UV-1208 spectrophotometer) using TE buffer as blank. The purity of the samples was determined from the ratio A_{260}/A_{280}.

2.11 Total RNA Isolation

2.11.1 Diethyl Pyrocarbonate (DEPC) Treatment

During RNA isolation all glassware and plastics should be treated by 0.01% DEPC solution overnight. Then, all glassware and plastics are autoclaved and dried in oven. Solutions that are used during the procedures should be DEPC treated or prepared with DEPC treated water.

Isolation of total RNA was done by RNeasy Kit supplied by Qiagen and according to rapid isolation method from gram negative bacteria (Reddy *et al*., 1990; Summers, 1970).

2.11.2 Isolation of Total RNA from Bacteria by RNeasy Mini Kit

In order to protect RNA from degradation during storage one volume of bacterial culture grown up to mid-log phase was added to two volumes of RNAprotect Bacteria Reagent into a reaction tube and the mixture was vortexed for 5 seconds immediately. Culture was incubated for 5 min at room temperature and centrifuged for 10 min at 6,500 rpm. The supernatant was decanted and the residual supernatant was removed by gently dabbing the inverted tube once onto a paper towel. Pellets were restored at -20 ˚C for up to 2 weeks or -70 ˚C for up to 4 weeks.

For the isolation of total RNA, the pellets of bacteria were thawed at room temperature. 200 µl of TE buffer containing lysozyme (Appendix A) was added and mixed by vortexing for 10 seconds. The mixture was incubated at room temperature for 5 minutes. During incubation, the tube was vortexed for 10 sec at least every 2 min. 700 µl of RLT (Appendix A) was added and vortexed vigorously. 500 µl of ethanol (96-100%) was added and the lysate was mixed by pipetting. Lysate containing ethanol was applied to RNeasy Mini Column in a 2 ml collection tube. Tube was closed gently and centrifuged for 15 sec at 13,000 rpm. The flow through was discarded. The maximum loading volume for the lysate is 700 µl. If the volume exceeds 700 µl, aliquots were loaded repeatedly to the columns. After adding 700 µl of Buffer RW1 (Appendix A) to the RNeasy column, the tube was centrifuged for 15 sec at 13,000 rpm to wash the column. The flow-through and the collection tube

were discarded and the RNeasy column was transferred into a new 2 ml collection tube. 500 µl of Buffer RPE (Appendix A) was pipetted onto the RNeasy column and centrifuged for 15 sec at 13,000 rpm. After discarding the flow-through, another 500 µl of RPE Buffer was added and centrifuged for 2 min at 13,000 rpm to allow the buffer to pass through the column and to dry the RNeasy silica-gel membrane. The column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 min. For elution, the column was transferred to a new 1.5 ml collection tube and 30-50 µl of RNase-free water was directly pipetted to the RNeasy silica-gel membrane. Finally, centrifugation was done for 1 min at 13,000 rpm and elution step was repeated using the first elute.

2.11.3 Rapid Isolation of RNA from Gram-Negative Bacteria

Cells from a 10-ml-gram negative bacteria culture grown up to mid-log phase was centrifuged for 10 minutes at 12,000xg, at 4 ˚C. Then, the cells were resuspended in 10 ml protoplasting buffer (Appendix A), 80 µl of 50 mg/ml lysozyme was added and incubated for 15 min on ice. Protoplasts were centrifuged for 5 min at 5,900xg, at 4 ˚C. The mixture was resuspended in 0.5 ml gram-negative lysing buffer (Appendix A), 15 µl of DEPC was added, mixed, and transferred to a microcentrifuge tube. After 5 min of incubation at 37 ˚C the mixture was chilled on ice. 250 µl of saturated NaCl was added, mixed, and incubated for 10 min on ice. Microcentrifuge was done for 10 min at 13,000 rpm at room temperature. The supernatant was transferred to two clean microcentrifuge tubes. After adding to each tube 1 ml ice cold 100% ethanol, the pellet was precipitated for 30 min on dry ice or overnight at -20 ˚C. Microcentrifuge was done for 15 min at 13,000 rpm, 4 ˚C. Then

the pellet was rinsed in 500 µl ice-cold 70% ethanol, dried and dissolved in100 µl DEPC-treated water.

2.11.4 Spectrophotometric Analysis of RNA

10 µl of DNA isolate was diluted with 990 µl of TE buffer (pH 8.0) in a quartz cuvette and the absorbances at 260 nm and 280 nm were measured (Shimadzu UV-1208 spectrophotometer) using TE buffer as a blank.

The purity of the samples was determined by taking the A_{260} reading to the A_{280} reading ratio. The value should be around 2.0 for pure RNA.

The quantity of the RNA samples was determined as:

RNA Concentration= A_{260} x 40 µg/ ml x Dilution Factor

Dilution factor was kept as 100 for all experiments.

2.11.5 Agarose Gel Electrophoresis of Total RNA

2.11.5.1 Denaturing Agarose Gel Electrophoresis

Agarose gel (1%) was prepared in 1X MOPS buffer (pH 7.0) (Appendix A) in a total volume of 25 ml. Formaldehyde was added (6% final concentration) for denaturing conditions (Kitlinska and Wojcierowski, 1995). 0.5 µg/ml EtBr (Appendix A) solution containing 0.1 M ammonium acetate was then added to the melted gel. 10 µl of total RNA was mixed with 1 µl of loading solution (Appendix A) and loaded onto the gel. After running for an hour at 70 V (Apelex PS-503 power supply), the RNA bands were visualized on a softwave UV transilluminator and photographed by using Vilber Lourmat Gel Imaging System.

2.11.5.2 Nondenaturing Agarose Gel Electrophoresis

1% of agarose gel was prepared with 25 ml 1X TAE Buffer (Appendix A). EtBR was added to give a 0.5 µg/ml final concentration to melted gel. 10 µl of total RNA isolate mixed with loading solution (Appendix A) was loaded onto the gel and the gel was run at 70 V for about one hour. RNA bands were visualized by UV and photographed.

2.12 DNase Treatment of Isolated RNAs

After isolation, RNAs were digested by DNase I with its buffer to get rid of genomic DNA contamination. 10 X DNase I buffer was included 100 mM Tris (pH 7.5), 25 mM $MgCl_2$ and 5 mM $CaCl_2$. 1 unit of DNase I was added to 1 µg of RNA and the reaction volume was completed to 10 µl. The mixture was incubated 15 minutes at room temperature and the reaction was stopped by adding 1 µl of 25 mM EDTA and heating 15 min at 65 ˚C.

2.13 Complementary DNA (cDNA) Preparation

0.1-5µg total RNA (1-10 µl) in a sterile PCR tube was added to 0.2µg random hexamer or 15-20pmol sequence-specific primer, then the volume was completed to 11µl with nuclease free sterile water. The mixture was incubated at 70°C for 5 min and chilled on ice. 4µl 5X reaction buffer and 2µl of 10mM dNTP mix (the final concentration 1mM) were added to the mixture. The volume was completed to 19µl with nuclease free sterile water. The mixture was incubated at 37°C for 5 min. If random primer was used, incubation was done at 25°C for 5 min. Then, 40 units of M-MuLV were added. The reaction mixture containing sequence-specific primer was incubated at 37°C for 60 min. If random hexamer primer was used, the mixture was

incubated at 25°C for 10 min and then at 37°C for 60 min. Finally, the reaction is stopped by heating at 70°C for 10 min and then chilled on ice for 1-2 min.

2.14 Polymerase Chain Reaction (PCR)

2.14.1 Primers Designed for Uptake Hydrogenase Genes

The primers designed for uptake hydrogenase genes were used to amplify both genomic DNA and cDNA fragments from *R. sphaeroides* O.U.001.

In order to amplify a specific region from the uptake hydrogenase mRNA, specific primers were needed. Primers for a reference gene were also needed as control. Constitutively expressed 16S rRNA was chosen as internal control.

In order to design a primer for uptake hydrogenase genes, sequence of another strain was used because there was no sequence available for *R.sphaeroides* O.U.001. Sequence of small subunit of hydrogenase genes of *R.sphaeroides* R.V. strain was obtained from the NCBI Genome Database. Then, the sequence was copied to "Oligos" software (Institute of Biotechnology, University of Helsinki) for the design of the primers. Listed results for the left and right primers were tested for self complementarities and primer-dimer formations. GC content of the primers was also important. Then, appropriate ones were selected and they were BLAST searched (NCBI Human Genome Database) in order to check if they are specific for *R. sphaeroides* or not. Finally, the selected pair of primers was synthesized by Iontek (Istanbul).

The primers that are used in this study were prepared by Iontek in Istanbul. The sequences are given in Table 2.2.

Table 2.2 Sequence of the primers for PCR amplifications

hupS	Left primer	5'-CAGCTTCATAAAATATTGCTCGC-3'
	Right primer	5'-TGTAGGTGATGACGCCGGTCA-3'
16S rRNA	Left primer	5'-CAGCTCGTGTCGTGAGATGT-3'
	Right primer	5'-TAGCACGTGTGTAGCCCAAC-3'
nif H	Left primer	5'- AACAAGGCGCAGGAAATCTA-3'
	Right primer	5'- ATACTGGATGACCGTCTCGC -3'

2.14.2 PCR Amplifications for Genomic DNA

PCR amplification reactions were performed by adding the following in the order into 0.5 ml Eppendorf tubes in a final volume of 50 µl. The PCR conditions and compositions are given in Table 2.3. After PCR amplifications completed, tubes were stored at -20 ˚C. Thermal cycler (Techne Techgene, England) was programmed as follows.

Table 2.3 PCR amplification ingredients, their final concentrations and PCR amplification conditions for genomic DNA of *R. sphaeroides* O.U.001

PCR Ingredients	Final Concentrations	PCR Amplifications Program	
Pyrogen free sterile water	To 50 µl	Initial denaturation at 94 ˚C for 10 min	
Reaction Buffer	1 X		
$MgCl_2$	1.5 mM	Denaturation at 94 ˚C for 1 min Annealing at 60 ˚C for 1 min Extension at 72 ˚C for 1 min	35 cycles
dNTP mix	0.2 mM each		
hupS left primer	0.2 µM		
hupS right primer	0.2 µM		
Genomic DNA template	100-300 ng	Final extension at 72 ˚C for 10 min Final hold at 4 ˚C indefinitely	
Taq DNA Polymerase	2.5 units/µl		

2.14.2.1 Extraction of PCR Products from Agarose Gel

After visualizing the PCR products on an agarose gel, the bands were cut from the gel and extracted for the products to be sequenced. DNA Extraction Kit (MBI Fermentas; including silica, washing buffer and DNA binding solution) was used. The gel slice that was exposed to UV minimally and containing the PCR product was excised from the gel and weighed (1 g app. equals to 1 ml). 3 volume of Binding Solution (Appendix A) was added to the 1 volume of gel and incubated for 5 min at 55 ˚C to dissolve agarose. 5 µl of silica powder suspension for up to 2.5 µg of DNA was added and incubation was done for 5 min at 55 ˚C. The mixture was vortexed for every 2 min to keep silica powder in suspension. Silica powder and DNA complex was spinned for 5 sec to form a pellet and the supernatant was removed. Then, 500 µl of ice cold wash buffer (Appendix A) was added, vortexed and spinned for 5 sec and supernatant was poured. This washing procedure was repeated for three times. During each washing, the pellet was resuspended completely. After the supernatant from the last wash was removed, the tube spinned again and the remaining liquid was removed by pipette. If necessary, the pellet was air-dried. After eluting DNA into water or TE buffer, the pellet was incubated for 5 min at 55 ˚C. Finally, the tube was spinned and the supernatant was removed into a new tube avoiding the pellet. The DNA fragment obtained was analyzed by agarose gel electrophoresis and the concentration was determined.

2.14.2.2 DNA Sequencing

The PCR amplified samples eluted from the agarose gel were sequenced by Iontek (Istanbul). Left and right primers designed to amplify 566 bp product from *R.*

sphaeroides R.V. were used for sequencing of small subunit of uptake hydrogenase gene of *R.sphaeroides* O.U.001. Obtained nucleotide sequence data was compared with the National Center of Biotechnology Information (NCBI) database using the BLAST search.

2.14.3 The Expression of Hydrogenase Genes

Regulation of expression of uptake hydrogenase genes of *R.sphaeroides* O.U.001 was examined under different metal ions by RT-PCR. Effect of two metal ions nickel and iron were studied at different concentrations. For this purpose, mediums containing different concentrations of metal ions were prepared and used for growth of bacteria (Table 2.1). After RNA isolation and cDNA preparation explained in section 2.11.2-2.11.3 and 2.12 PCR amplifications were done.

2.14.3.1 The Multiplex-PCR

Multiplex PCR employs different primer pairs in the same amplification reaction. Amplification reactions contents, their final concentrations and amplification programs are listed in Table 2.4. The program was the same with the program for genomic DNA amplifications.

Table 2.4 The Multiplex-PCR amplification

PCR Ingredients	Final Cncentrations	PCR Amplifications Program	
Pyrogen free sterile water	to 50 µl	Initial denaturation at 94 ˚C for 10 min	
Reaction Buffer	1 X		
$MgCl_2$	1.5 mM	Denaturation at 94 ˚C for 1 min Annealing at 60 ˚C for 1 min Extension at 72 ˚C for 1 min	35 cycles
dNTP mix	0.2 mM each		
hupS left primer	0.2 µM		
hupS right primer	0.2 µM		
16S rRNA left primer	0.2 µM		
16S rRNA right primer	0.2 µM		
cDNA template	100-300 ng	Final extension at 72 ˚C for 10 min Final hold at 4 ˚C indefinitely	
Taq DNA Polymerase	2.5 units/µl		

The products of PCR amplifications were expected to be 170 bp (16S rRNA) and 566 bp (*hupS*). The primers indicated as left primers are the sense primers, right primers are the anti-sense primers.

2.14.4 Agarose Gel Electrophoresis of PCR Products

As PCR products were analyzed by 1.5 % agarose gels in a total volume of 40 ml, 1.5% of agarose and 1 X TAE Buffer (Appendix A) was prepared. EtBr was added to a final concentration of 0.5 µg/ml. 6 µl of DNA ladder (80-1031 bp) mixed with loading dye (Appendix A) was loaded to the first well. 15 µl of PCR products mixed with loading dye (3 µl) was loaded to the wells and the gel was run for 60 min at 90 V. The bands were visualized under UV and photographed.

2.14.5 Densitometric Analysis of PCR Products

The gel photographs of the Multiplex PCR gels were processed with the Software named BioProfil Bio-1D V99.04 Vilber-Lourmat. The areas under the peaks were calculated by the software and expression analysis was carried out.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Growth of Bacteria

Photosynthetic bacterium exhibits remarkable metabolic diversity. This bacterium is capable of growth by aerobic and anaerobic respiration, fermentation, and anoxygenic photosynthesis. The bacteria can grow heterothrophically as well as autotrophically. *R. sphaeroides* O.U.001 produce hydrogen when grown heterotrophically under illumination in the presence of argon gas. When growing chemoheterotrophically, growth of *R.sphaeroides* is supported by aerobic respiration. When oxygen is removed from such a culture, a series of events is triggered which results in differentiation of the cytoplasmic membrane through a process of invagination into specialized domains which comprise the photosynthetic intracytoplasmic membrane system (ICM). The ICM is physically continuous with the cytoplasmic membrane but structurally and functionally distinct in that the ICM specifically contains all of the membrane components required for the light reactions of photosynthesis. Therefore changing the conditions may take some time to adapt for the bacteria (Kiley *et al*., 1988).

3.2 Effect of different metal ions on growth profiles of *Rhodobacter sphaeroides* O.U.001

Since hydrogenases are metalloenzymes, different sets of growth medium containing different concentrations of nickel, iron and molybdenum listed in Table 2.1 were used for examining growth of *R. sphaeroides* O.U.001 (Section 1.7., 10). The graphs obtained in growth studies are given in Figure 3.1 through Figure 3.3. Growth of *R. sphaeroides* O.U.001 was observed as a change of absorbance at 660 nm measured at time intervals. As can be seen from the figures, growth was seem not to be affected significantly by different concentrations nickel, molybdenum and iron. There was a slightly increase in cultures grown in Fe 10X medium but this increase is not important.

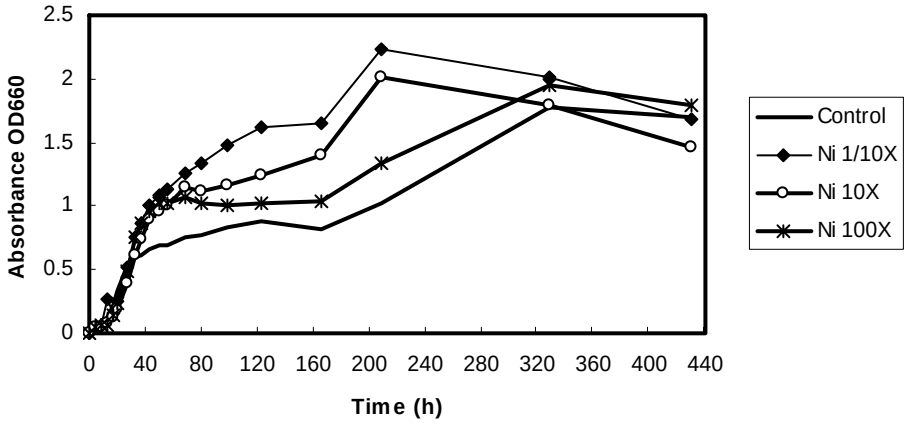

Figure 3.1 Growth of *Rhodobacter sphaeroides* O.U.001 in different concentrations of Nickel containing medium

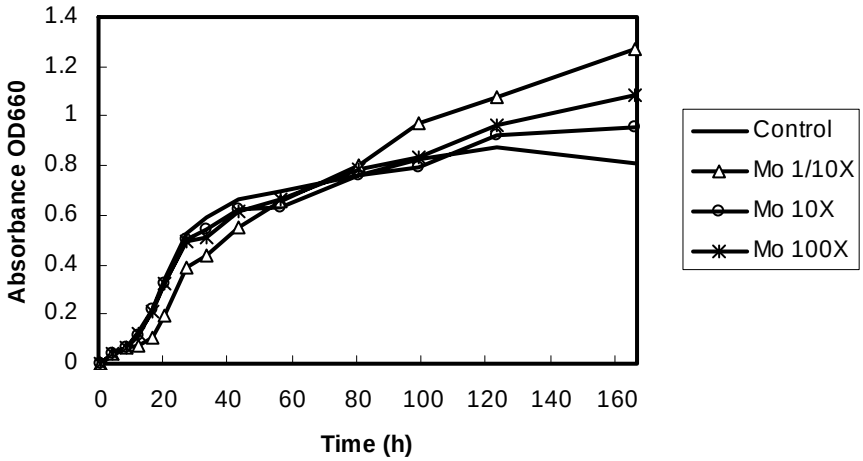

Figure 3.2 Growth of *Rhodobacter sphaeroides* O.U.001 in different concentrations of Molybdenum containing medium

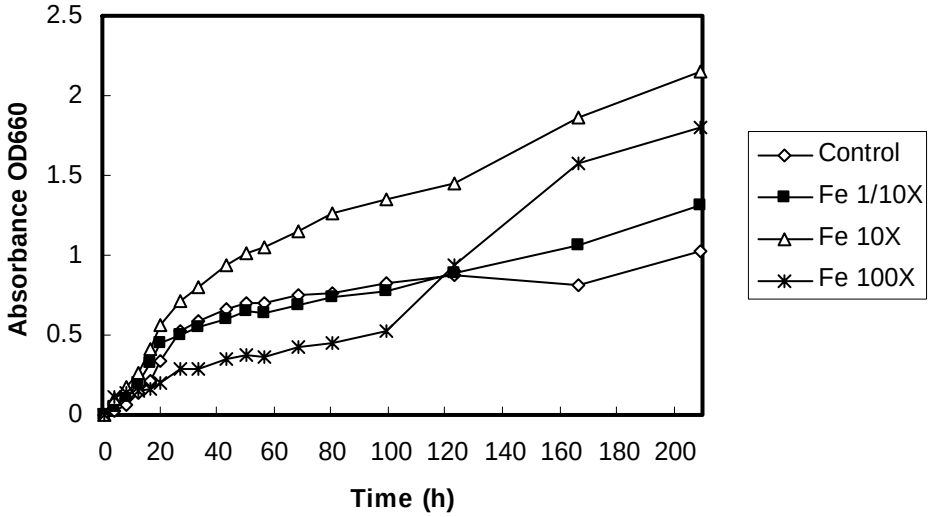

Figure 3.3 Growth of *Rhodobacter sphaeroides* O.U.001 in different concentrations of Iron containing medium

3.2.1 Identification of Uptake Hydrogenase Gene in *R. sphaeroides* O.U.001

Although the hydrogenase genes from different organisms have been described, transcripts of the hup genes have been rarely studied for Rhodobacter species.

In order to design a primer for uptake hydrogenase genes sequence of *R. sphaeroides* R.V. strain was used because there was no available sequence for *R. sphaeroides* O.U.001 (Section 1.13.2).

3.2.1.1 Polymerase Chain Reactions of Genomic DNA

After isolating genomic DNA from bacteria as mentioned in Section 2.10, PCR amplifications using primers designed for small subunit of uptake hydrogenase (*hupS*) were carried out. The product of PCR observed by running agarose gel electrophoresis was 566 bp (Figure 3.4).

Since Mg^{2+} ions form complexes with dNTPs, primers and DNA templates, the optimal concentration of $MgCl_2$ has to be optimized. High Mg^{2+} level increases the yield of non-specific products and too low Mg^{2+} results in low yield of PCR products.

The primers used in this work have a high GC content (69 %), therefore denaturation time was increased to 10 min and 5% DMSO was used to facilitate the denaturation of DNA. Since DMSO is known to inhibit *Taq* DNA Polymerase approximately 50 %, the amount of the enzyme was increased to 4-5 units/ µl.

Annealing temperature of the process was optimized. The annealing temperature was increased stepwise by 1-2 ˚C until getting rid of non-specific PCR products.

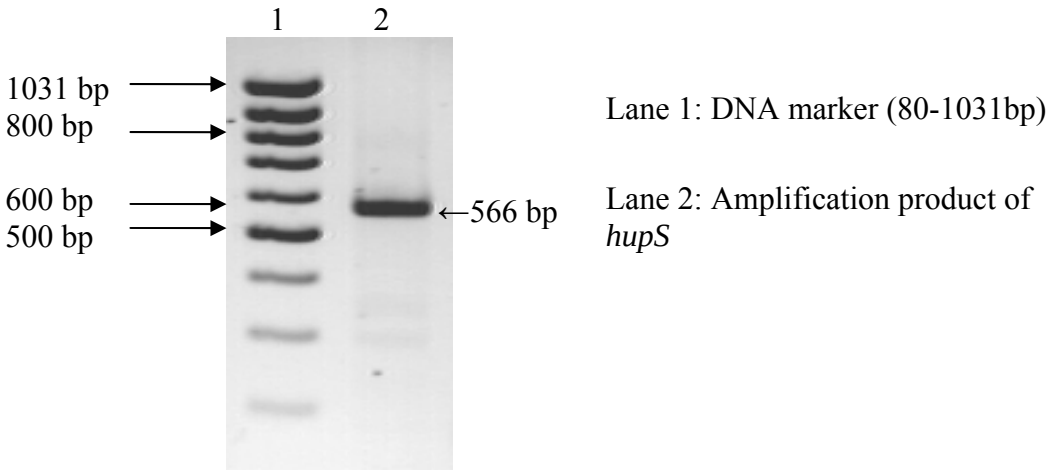

Figure 3.4 Agarose gel electrophoresis of PCR products

3.2.1.1.1 Sequence Analysis of PCR Products

After PCR amplifications and gel electrophoresis PCR products were recovered from agarose gel (Section 2.13.3.1.) and DNA was sequenced by Iontek (Istanbul). Obtained nucleotide sequence data was compared with the National Center of Biotechnology Information (NCBI) database using the BLAST search and the homology was found to be 93 % with *R. sphaeroides* RV strain (Figure 3.5 and Figure 3.6; Appendix).

Identities=146/156 (93%), Gaps=3/156 (1%)

```
36    tgatcggcttgctcgaggatgaccttgatgcaccggca-cgcccgcgtcgggttgggcgc
      |||||||||||| |||||||||||||||| |||||||||  ||||||||||||||||||| ||
7533  tgatcggcttg-tcgaggatgaccttg-tgcaccggcgtcgcccgcgtcgggttgggtgc

      cgccgcctgcacgcagccgtacgaggcgcaggcgccccagctgatgatggccttggcatg
      |||||||||||||||||||| |||||||||| |||||||||||||||||||||||||||
      cgccgcctgcacgcagccgtaggaggcgcaggccccccagctgatgatggccttggcatg


      ctcggcggccatcttcagctgctccacgaagggctt 190 (R.sphaeroides O.U.001)
      ||||| |||||||||||||| ||||||||||||||
      ttcggccgccatcttcagctgttccacgaagggctt 7380 (R.sphaeroides R.V)
```

Figure 3.5 Sequence analysis results of *R. sphaeroides* O.U.001 using *HupS* left primers

Rhodobacter sphaeroides uptake hydrogenase operon
Identities = 269/293 (91%), Gaps = 3/293 (1%)

```
17    cgggcagcccgggccctgtatgatcggcttgtcgaggatgaccttgatgcaccggca-cg
      ||||||||||||  |||  |||||||||||||||||||||||||| |||||||||  ||
7553  cgggcagcccggcaccttgatgatcggcttgtcgaggatgaccttg-tgcaccggcgtcg


      cccgcgtcgggttgggcgccgccgcctgcacgcagccgtangaggcgcaggcgccccagc
      |||||||||||||||| |||||||||||||||||||||| |||||||||| |||||||
      cccgcgtcgggttgggtgccgccgcctgcacgcagccgtaggaggcgcaggcccccccagc


      tgatgatggccttggcatgctcggcggccatcttcagctgctccacgaagggcttgccgc
      |||||||||||||||||| ||||| |||||||||||||| ||||||||||||||||||||
      tgatgatggccttggcatgttcggccgccatcttcagctgttccacgaagggcttgccgc


      cgatgatgcaatacatcccgtcctcgttcagcggcgggttgccctcgaccgcgaggatgt
      |||||||||||||||||||||||||||||||||||||||||||||||||||| || || ||||
      cgatgatgcaatacatcccgtcctcgttcagcggcgggttgccctcgacggcaagaatgt


      atttgtccttgtatntctcgaatcgtgtccatcatcgcctcttcggcctgatg 308
      | ||| |||||||| ||||| |||||||||||||| |||  |||| ||||||||
      agttgcccttgtatttctcg-atcgtgtccatcagcgcggcttccgcctgatg 7263
```

Figure 3.6 Sequence analysis results of *R. sphaeroides* O.U.001 using *HupS* right primers

3.3 Hydrogen Production at Different Metal Ion Concentrations

R. sphaeroides O.U.001 was grown in Biebl and Pfenning medium composed of 15 mM/2 mM malate to glutamate ratio and different concentrations of nickel and iron (Table 2.1). During hydrogen production tests, 55 ml penicillin bottles were used and hydrogen production and pH curves were obtained (Figure 3.7 through Figure 3.10). The experimental data is given in Appendix B.

3.3.1 Hydrogen Production at Different Concentrations of Nickel Ion

The biosynthesis of NiFe hydrogenases is closely related to nickel metabolism. Nickel effects both the formation of an active enzyme and the transcriptional level of the hydrogenase gene. Addition of external nickel to the medium increased the hydrogenase uptake activity in several bacteria (Daday *et al*., 1985, Kumar *et al*., 1991, Oxelfelt *et al*., 1995, Xiankong *et al*., 1984). Figure 3.7 shows the hydrogen production of *R. sphaeroides* O.U.001 at different concentrations of Nickel. Hydrogen gas production was started about 15-20th hours and continued to about 90th hour at various concentrations of Ni^{++} containing medium. Among all media that contained different concentrations of nickel ion, the maximum hydrogen gas production was achieved in Ni 100X media (8.4 μM). The addition of Ni^{++} to the culture had a profound effect to the net H_2 production. Increase of Nickel ion concentration 100 times increased H_2 production about 16.85% compared to the normal medium. Total H_2 production was about 60 ml. Gas Chromatography (GC) analysis of the gas showed that about 98% of total gas was H_2.

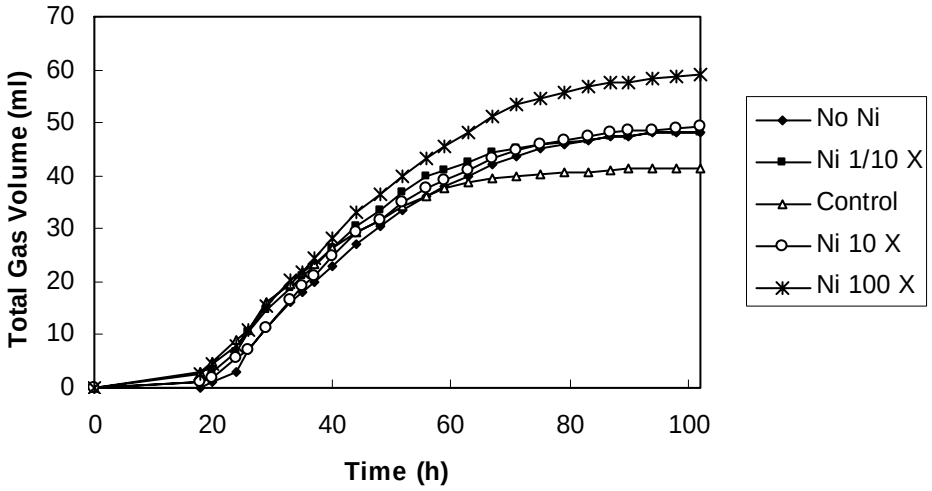

Figure 3.7 Hydrogen gas production at various concentrations of nickel ion

3.3.2 Hydrogen Production at Various Concentrations of Iron Ion

Figure 3.8 shows the hydrogen production of *R. sphaeroides* O.U.001 at different concentrations of iron. Mediums containing different concentrations of iron showed different pattern of hydrogen productions. No Fe, 0.1X Fe, control and 10X medium cultures started to produce hydrogen gas at about 20th hours and continued until about 120th hours. Control medium culture and 0.1X Fe medium cultures showed similar hydrogen production rates and their maximum produced hydrogen gas was about 37-40 ml. After 40th hour, 10X Fe medium showed a dramatic increase in hydrogen production and continued until 120th hour achieving the maximum hydrogen production of about 60 ml. Although 50X Fe medium containing culture start producing hydrogen gas at about 20th hours, the hydrogen gas produced was less than the other medium. In can be hypothesized that because at high concentrations the color of the medium was darker (dark yellow), light might be insufficient.

Therefore the light could not reach homogenously to the medium. The maximum gas production of this medium was about 22 ml. Culture that was not contained iron could not start hydrogen production until 80th hour and produced about 30 ml of hydrogen maximally.

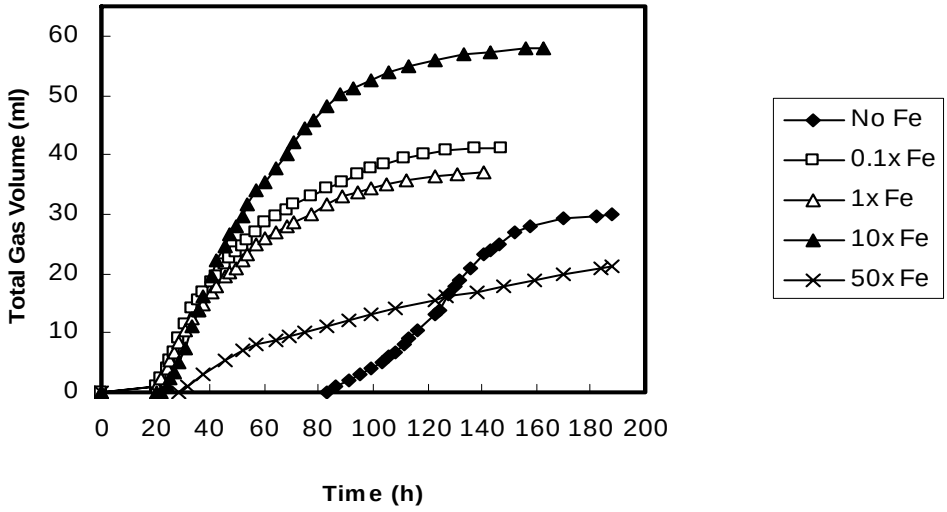

Figure 3.8 Total volume of hydrogen gas production for various concentrations of Iron

3.3.3 pH Changes During Growth of *R. sphaeroides* O.U.001 at Different Concentrations of Metal Ions

It is known that growth of *R. sphaeroides* O.U.001 occurs around pH 6.0 and 8.5 (optimum pH is about 6.8). Also it has been shown that nitrogenase activity was maximal at a pH range of 6.5 to 7.0. Therefore hydrogen production can be negatively affected by increasing pH. Figure 3.9 and Figure 3.10 show the pH change of the Biebl and Pfenning medium during hydrogen production at different concentrations of metal ions. Because in the early hydrogen production experiments increase in pH of the medium interrupted the hydrogen production, amount of buffer

has been increased by two folds. Initial pH values were adjusted to about 6.8. Until the production of hydrogen started, increase of pH continued. The increase in the pH of the medium during hydrogen gas production had an inhibitory effect so the pH of the medium was maintained around 7.0 by increasing the buffer capacity. After the hydrogen production started, pH values slightly decreased as a result of fermentation. All of the mediums that contained different concentrations of nickel showed similar pH changes. In the case of mediums with different concentrations of iron, only the medium which doesn't contain iron showed fluctuation in pH values since in this medium hydrogen production did not start until about 80 h where pH value increased to 9.2. However, pH decreased gradually after the H_2 production started.

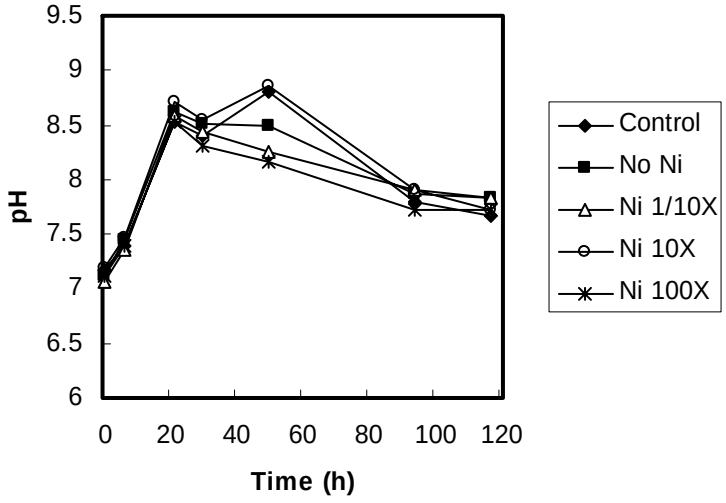

Figure 3.9 pH change during growth of bacteria at different concentrations of Nickel

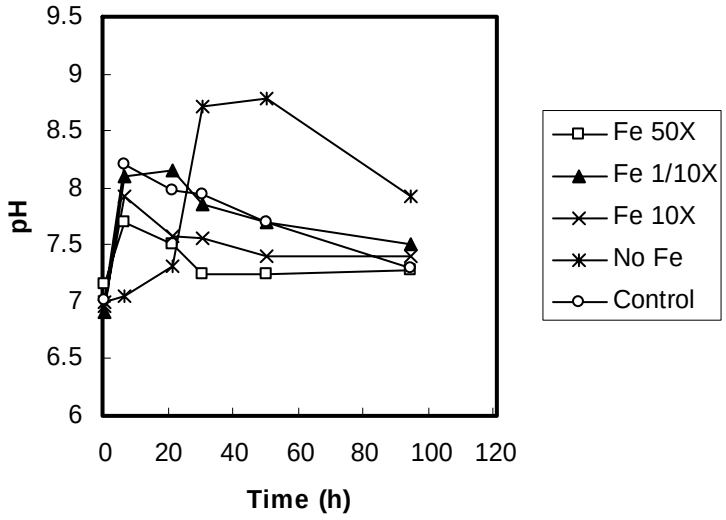

Figure 3.10 pH change during growth of bacteria at different concentrations of iron ion

3.4 Expression of Uptake Hydrogenase Genes at Different Concentrations of Metal Ions in *R.sphaeroides* O.U.001

Isolated RNAs from different concentrations of nickel and iron containing media was reverse transcribed to cDNA and utilized in PCR analysis for examining the expression of uptake hydrogenase genes. During RT-PCR amplifications, same primer pairs and the same program were used with PCR of genomic DNA (Table 2.2). After several optimization of $MgCl_2$ concentrations, dNTP concentrations and annealing temperature, the products were obtained successfully. Obtained products were observed by 1.5 % agarose gel electrophoresis. Figure 3.13 through Figure 3.16 show the RT-PCR amplification products.

3.4.1.1 Isolation of Total RNA from *R. sphaeroides* O.U.001

To check the integrity of RNA isolated from bacteria as mentioned in Section 2.11, agarose gel electrophoresis was performed. The gel photograph of total RNA extracts was given in Figure 3.11. Since rRNAs are the predominating species of RNA, only rRNA bands are expected to be seen as a result of gel electrophoresis of total RNA. In prokaryotes there are 23S, 16S and 5S rRNAs. Figure 3.11 shows that there are two clear bands of rRNA; 16S RNA and 23S RNA. When the rRNA bands are observed intact, the mRNAs are also assumed to be intact.

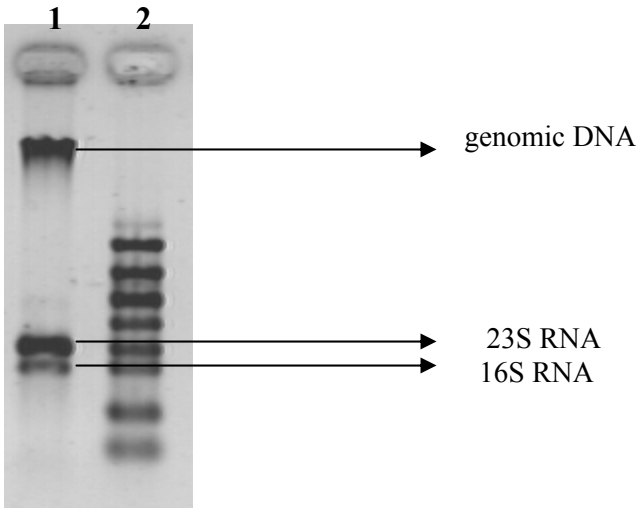

Figure 3.11 Agarose gel electrophoresis of total RNA extract from sample
Lane 1: total RNA
Lane 2: RNA Ladder (200-6000 bases)

3.4.1.2 Spectrophotometric Analysis of RNA

Spectrophotometric analysis were performed for all RNA samples obtained. The absorbance readings at 260 nm and 280 nm were used to calculate the quantity

and the purity of the samples as described in Section 2.11.4. The results are given in Table 3.1 and

Table 3.2.

Table 3.1 Spectrophotometric analysis result of RNA samples through time at various Nickel concentrations

Medium	Time after inoculation (hour)	A_{260}	A_{280}	A_{260}/A_{280}	RNA Concentration (µg/ µl)
No Nickel	22	0.048	0.030	1.60	0.192
	46	0.112	0.070	1.60	0.448
	105	0.049	0.026	1.88	0.196
Control (0.084 µM Ni^{++})	22	0.116	0.063	1.84	0.464
	46	0.115	0.062	1.85	0.460
	105	0.066	0.047	1.40	0.264
Nickel 100X (8.4 µM Ni^{++})	22	0.075	0.045	1.67	0.300
	46	0.109	0.069	1.58	0.436
	105	0.203	0.124	1.64	0.812

Table 3.2 Spectrophotometric analysis result of RNA samples through time at various Ferric citrate concentrations

Medium	Time after inoculation (hour)	A_{260}	A_{280}	A_{260}/A_{280}	RNA Concentration (µg/ µl)
No Iron	12	0.070	0.042	1.67	0.280
	30	0.118	0.082	1.44	0.472
	52	0.075	0.043	1.74	0.300
	120	0.067	0.046	1.46	0.268
Control (0.1 mM Ferric-citrate)	12	0.316	0.212	1.49	1.264
	30	0.080	0.056	1.43	0.320
	52	0.090	0.062	1.45	0.360
	120	0.083	0.053	1.57	0.332
Iron 10X (1 mM Ferric-citrate	12	0.225	0.145	1.55	0.900
	30	0.145	0.089	1.63	0.580
	52	0.094	0.070	1.34	0.376
	120	0.139	0.078	1.78	0.556

The spectrophotometric analysis showed that most of the RNA samples have good ratio of A_{260}/ A_{280} close to 2.0 which suggests purity. Lower ratios imply that there might be both DNA and protein contaminations in the samples. For the RNA samples that can be utilized in RT-PCR, the ratio should be at least 1.4. Therefore the samples are suitable for RT-PCR studies. Different values of A_{260} for the RNA samples might be the result of different starting amount of cultures or experimental errors during the process, although all of the samples were treated same way.

Because prokaryotic DNAs have no introns primers designed for RT-PCR will amplify cDNA generated from mRNA as well as the genomic sequences. Although the genomic DNA contamination is not critical for eukaryotic RT-PCR this is not the case with prokaryotic RT-PCR studies. In this case all genomic DNA must be excluded from the samples.

3.4.1.3 Optimization of DNase I Treatment of RNA Samples

Since pure RNA samples are necessary for RT-PCR amplifications, after isolation of total RNA, samples were treated with different concentrations of RNase free DNase I to get rid of genomic DNA of bacteria as mentioned before (Section 2.12). For the inactivation of DNase I, EDTA was used to chelate the magnesium ions in the DNase I reaction. EDTA is used during the inactivation of DNase I because at 65 ˚C free divalent metal ions can promote RNA hydrolysis.

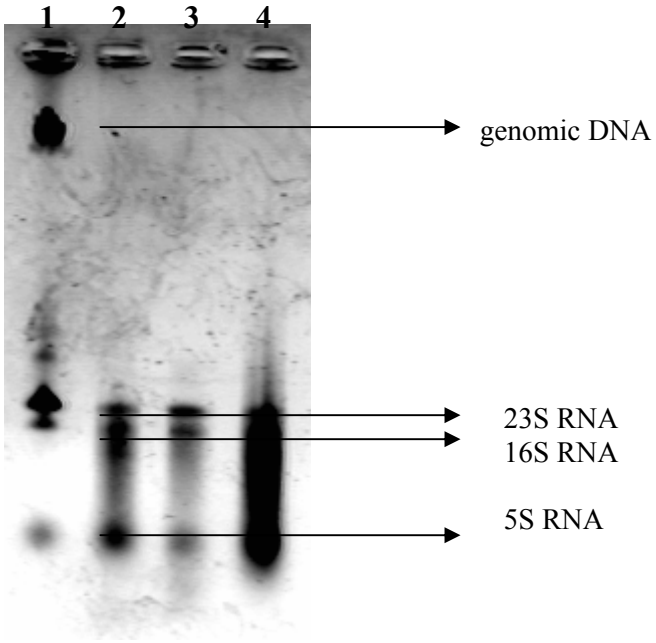

Figure 3.12 Agarose gel analysis of RNA samples treated with different amounts of DNase I

Lane 1: 2 μg RNA not treated with DNase I
Lane 2: 2 μg RNA treated with 1 Unit of DNase I
Lane 3: 2 μg RNA treated with 1.5 Units of DNase I
Lane 4: 2 μg RNA treated with 2 Units of DNase I

After treating RNA samples with different concentrations of DNase I, optimum concentrations have been found. 1 Unit of DNase I was not sufficient for complete digesting of genomic DNA and 2 Units of DNase I was too much, since it also digested RNA samples. 1.5 Units of DNase I was found to be the optimum amount for digesting 2 μg of RNA samples (Figure 3.12).

3.4.1.4 Time Dependent *hupS* Expression at Different Nickel Concentrations

After analyzing hydrogen production of *R. sphaeroides* O.U.001 at different concentrations of external Ni^{++}, three Ni^{++} concentrations were chosen for expression studies of *hupS* genes; medium containing no Nickel, control medium (0.084 μM

Ni^{++}), and Ni 100X medium (8.4 μM Ni^{++}). After cells were inoculated to selected mediums, samples were taken at time intervals and RT-PCR was performed. Hydrogen production of the cultures was also examined throughout the study. Sampling time was selected according to amount of produced hydrogen;

a) Right after hydrogen production started (22^{th} hour after inoculation)

b) Maximum hydrogen production (46^{th} hour after inoculation)

c) After hydrogen production was ended (105^{th} hour after inoculation) (Figure 3.13)

Amount of cells from the bacterial cultures were adjusted to be the same for all samples (OD_{660} was about 0.6). After isolation of RNA, cDNAs were prepared and RT-PCR was done using *hupS* primers and 16S rRNA internal control primes. Figure 3.13 shows time dependent RT-PCR amplification products of *hupS* primers visualized on 1.5% agarose gel.

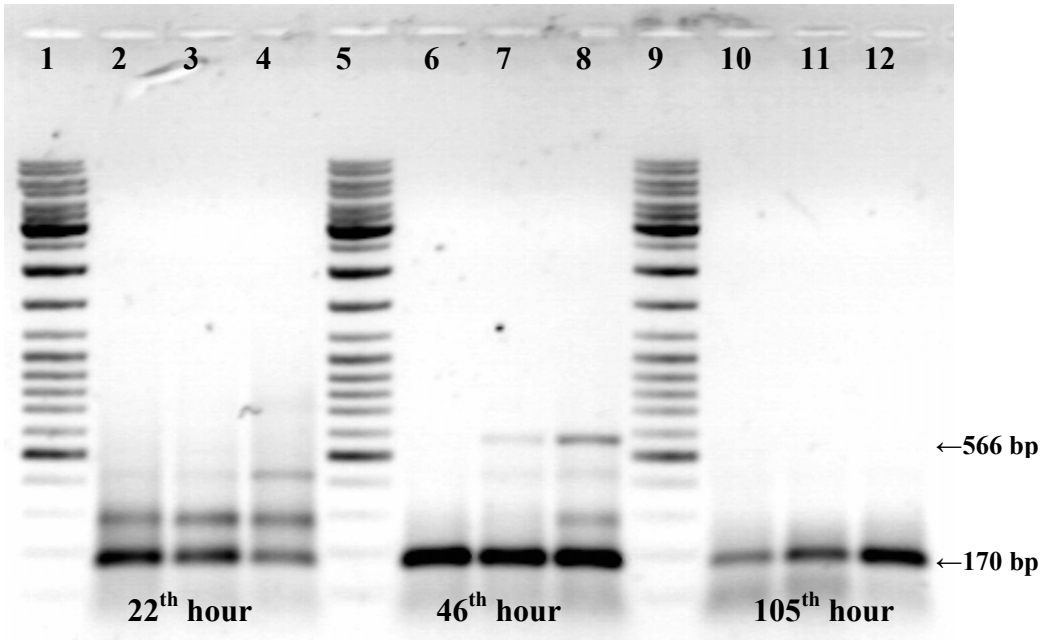

Figure 3.13 Analysis of RT-PCR amplification products of *hupS* primers visualized on 1.5 % agarose gel

Lane 1, 5, 9: DNA markers
Lane 2-4: Samples after 22 hours of inoculation
Lane 2: Samples taken from without Nickel Medium
Lane 3: Samples taken from control (0.084 μM Ni) Medium
Lane 4: Samples taken from Nickel 100 X (8.4 μM Ni) Medium
Lane 6-8: Samples after 46 hours of inoculation
Lane 6: Samples taken from without Nickel Medium
Lane 7: Samples taken from control (0.084 μM Ni) Medium
Lane 8: Samples taken from Nickel 100 X (8.4 μM Ni) Medium
Lane 10-12: Samples after 105 hours of inoculation
Lane 10: Samples taken from without Nickel Medium
Lane 11: Samples taken from control (0.084 μM Ni) Medium
Lane 12: Samples taken from Nickel 100 X (8.4 μM Ni) Medium
16 S rRNA primers were used as an internal control (170 bp)

There were no PCR products obtained for *hupS* primers at 22^{th} and 105^{th} hours. No expression was observed at no Ni^{++} medium, however the expression of *hupS* gene was about 6 folds at 100X Ni^{++} concentration at 46^{th} hour (Figure 3.14). It was thought that for *hupS* genes to be expressed, hydrogen concentration must reach

to a certain level. Therefore there were no PCR products for *hupS* primers at 22th hour after inoculation.

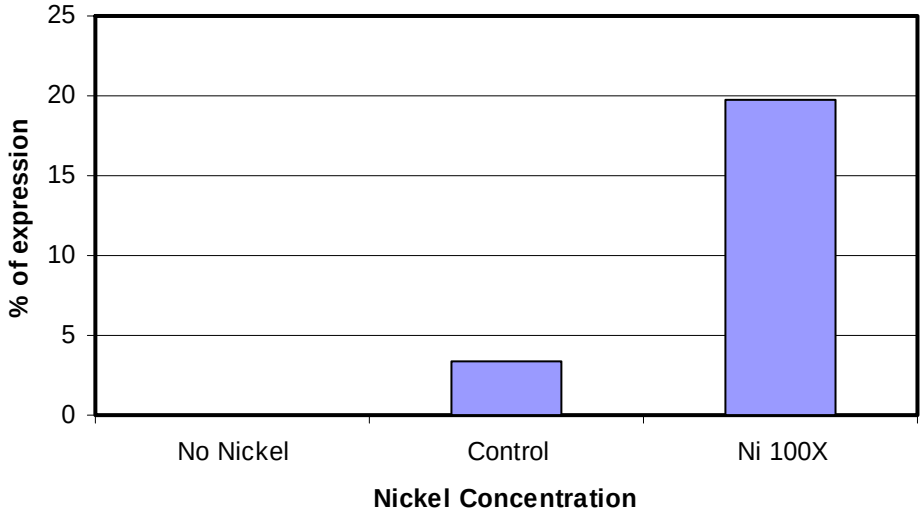

Figure 3.14 Densitometric analysis of *R.sphaeroides* O.U.001 *hupS* gene expression at different nickel concentrations

Addition of nickel to the growth medium increased the hydrogen uptake activity in several cyanobacteria (Daday *et al*., 1985; Kumar *et al*., 1991; Oxelfelt *et al*., 1995; Xiankong *et al*., 1984). In this study, where addition of 8.4 µM of nickel increased the in vivo hydrogen uptake by *R. sphaeroides* O.U.001 demonstrated an increase in expression of *hupS* genes. The results are in agreement with previous studies involving *N. muscorum* and *Nostoc* sp. strain PCC 73102 (Axelsson *et al.*, 2002).

3.4.1.5 Time Dependent *hupS* Expression at Different Iron Concentrations

After analyzing hydrogen production of *R.sphaeroides* O.U.001 at different concentrations of Ferric-citrate, three Ferric-citrate concentrations were chosen for

expression studies of *hupS* genes; medium containing no Iron, control medium (0.1 mM Ferric-citrate), and Fe 10X medium (1 mM Ferric-citrate). After cells were inoculated to three selected mediums, samples were taken through time and RT-PCR was done. Hydrogen production of the cultures was also examined throughout the study. Sampling time was selected according to amount of produced hydrogen;

a) Before hydrogen production started (12^{th} hour after inoculation)

b) Right after hydrogen production started (30^{th} hour after inoculation)

c) Maximum hydrogen production region (52^{th} hour after inoculation)

d) After hydrogen production was ended (120^{th} hour after inoculation) (Figure 3.15)

After isolation of RNA, cDNAs were prepared and RT-PCR was done using *hupS* primers and 16S rRNA primes. Figure 3.15 shows time dependent RT-PCR amplification products of *hupS* primers visualized on 1.5% agarose gel.

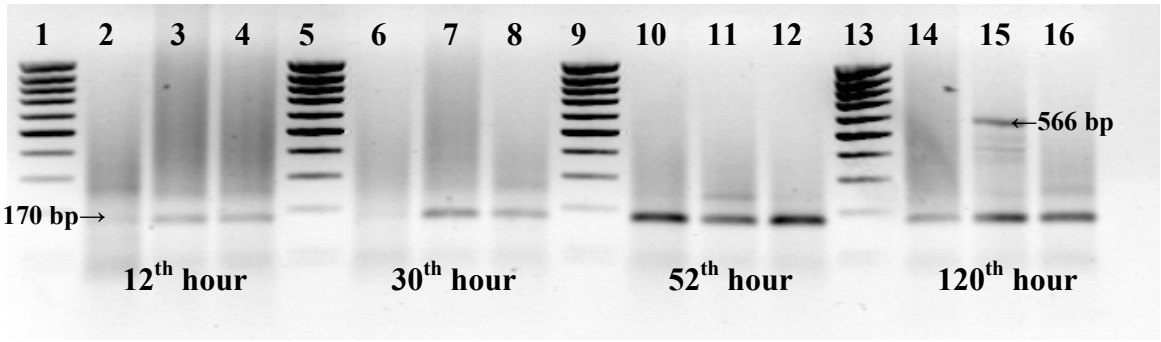

Figure 3.15 Analysis of RT-PCR amplification products with *hupS* primers visualized on 1.5 % agarose gel

Lane 1, 5, 9, 13: DNA markers
Lane 2-4: Samples after 12 hours of inoculation
- Lane 2: Samples taken from without Iron Medium
- Lane 3: Samples taken from control (0.1 mM Fe) Medium
- Lane 4: Samples taken from Iron 10 X (1 mM Fe) Medium

Lane 6-8: Samples after 30 hours of inoculation
- Lane 6: Samples taken from without Iron Medium
- Lane 7: Samples taken from control (0.1 mM Fe) Medium
- Lane 8: Samples taken from Iron 10 X (1 mM Fe) Medium

Lane 10-12: Samples after 52 hours of inoculation
- Lane 10: Samples taken from without Iron Medium
- Lane 11: Samples taken from control (0.1 mM Fe) Medium
- Lane 12: Samples taken from Iron 10 X (1 mM Fe) Medium

Lane 14-16: Samples after 120 hours of inoculation
- Lane 14: Samples taken from without Iron Medium
- Lane 15: Samples taken from control (0.1 mM Fe) Medium
- Lane 16: Samples taken from Iron 10 X (1 mM Fe) Medium

16 S rRNA primers were used as an internal control (170 bp)

No expression of *hupS* genes was observed until 120th hour after inoculation. There was a PCR product with *hupS* primers for culture containing 0.1 mM Ferric-citrate (control medium) after hydrogen production stopped. *hupS* genes might not be expressed until hydrogen concentration reaches to a certain level.

Time dependent *hupS* expression at different iron concentrations was not determined before in literature. Further studies should be carried out to confirm the importance of iron concentrations for uptake hydrogenase expression.

3.4.1.6 Time Dependent *nifH* Expression at Different Iron Concentrations

After examining time dependent RT-PCR of *hupS* genes at different Fe-citrate concentrations, time dependent *nifH* expression was studied with the same samples. RT-PCR was done by using *nifH* primers and cDNAs were the same with the ones that was used for *hupS* expression. Figure 3.16 shows RT-PCR amplifications with *nifH* primers.

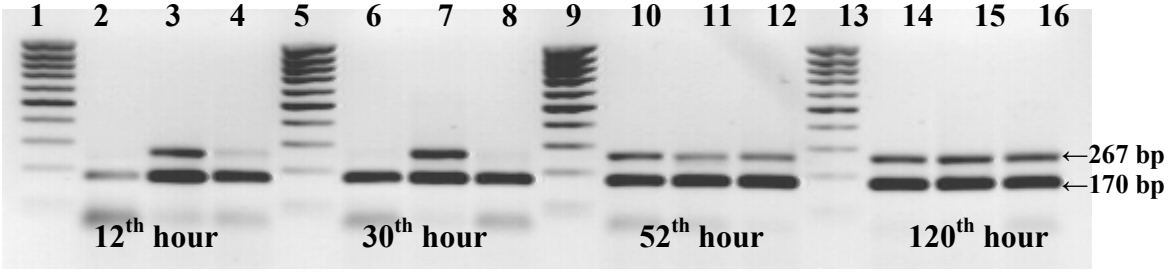

Figure 3.16 Analysis of RT-PCR amplification products with *nifH* primers visualized on 1.5 % agarose gel

Lane 1, 5, 9, 13: DNA markers
Lane 2-4: Samples after 12 hours of inoculation
- Lane 2: Samples taken from without Iron Medium
- Lane 3: Samples taken from control (0.1 mM Fe) Medium
- Lane 4: Samples taken from Iron 10 X (1 mM Fe) Medium

Lane 6-8: Samples after 30 hours of inoculation
- Lane 6: Samples taken from without Iron Medium
- Lane 7: Samples taken from control (0.1 mM Fe) Medium
- Lane 8: Samples taken from Iron 10 X (1 mM Fe) Medium

Lane 10-12: Samples after 52 hours of inoculation
- Lane 10: Samples taken from without Iron Medium
- Lane 11: Samples taken from control (0.1 mM Fe) Medium
- Lane 12: Samples taken from Iron 10 X (1 mM Fe) Medium

Lane 14-16: Samples after 120 hours of inoculation
- Lane 14: Samples taken from without Iron Medium
- Lane 15: Samples taken from control (0.1 mM Fe) Medium
- Lane 16: Samples taken from Iron 10 X (1 mM Fe) Medium

16 S rRNA primers were used as an internal control (170 bp)

0.1 mM Fe seems to be the optimal concentration for the expression of *nifH* genes. At higher and lower Fe concentrations no *nifH* expression was observed up to

30th hour. After that *nifH* expression was observed in all iron concentrations. It may be suggested that Fe concentration may be critical at the early stages of hydrogen production however, when hydrogen gas was accumulated in the system the expression of nitrogenase genes become insensitive to iron levels.

CHAPTER 4

CONCLUSION

- The presence of the uptake hydrogenase genes was shown in *R.sphaeroides* O.U.001 strain for the first time and *hupS* gene sequence was determined. The sequence shows 93% of homology with the uptake hydrogenase *hupS* of *R.sphaeroides* R.V.
- Concentrations of metal ions nickel, molybdenum and iron in growth media does not seem to effect the growth of the bacteria considerably
- The maximum hydrogen gas production was achieved at 8.4μM of nickel and 0.1 mM of iron containing media.The expression of uptake hydrogenase genes were examined by RT-PCR. Increasing the concentration of Ni^{++} up to 8.4μM increased the expression of uptake hydrogenase genes (*hupS*).
- At varied concentrations of Fe-citrate (0.01 mM-0.1 mM) expression of *hupS* was not detected until hydrogen production stopped. *hupS* genes might not be expressed until hydrogen concentration reaches to a certain level.
- 0.1 mM Fe seems to be the optimal concentration for the expression of *nifH* genes. At higher and lower Fe concentrations no *nifH* expression was observed up to 30th hour. Fe concentration may be critical at the early stages of hydrogen production. However when hydrogen gas reached to a certain

level, the expression of nitrogenase genes (*nifH*) become insensitive to iron levels.

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APPENDIX A

MEDIA COMPOSITION AND PREPARATION

Culture Media with Different L-Malic Acid to Na-Glutamate Molar Ratios

Media that are going to be used for growth (7.5/10; L-Malic acid/Na Glutamate, mM/mM) and for hydrogen production (15/2; L-Malic acid/Na Glutamate, mM/mM) are shown in Table A1.

Table A1. Composition of Culture Media with Different L-Malic Acid to Na Glutamate Molar Ratios

Medium Composition (g/L)	L-Malic acid/Na Glutamate 7.5/10 (mM/mM)	L-Malic acid/Na Glutamate 15/2 (mM/mM)
KH_2PO_4	0.5	0.5
$MgSO_4.7H_2O$	0.2	0.2
NaCl	0.4	0.4
Na Glutamate	1.8	0.36
$CaCl_2.2H_2O$	0.05	0.05
L-Malic Acid	1.0	2.0
Vitamin Solution	1 ml	1 ml
Trace Element Solution 7	1 ml	1 ml
Fe-Citrate Solution	5 ml	5 ml

The solid ingredients were weighed and dissolved in 1000 ml of distilled water. Instead of 6 ml of removed solution, trace element and Fe-citrate solutions were added. pH of the solution was adjusted to 6.8- 7.0 by adding NaOH. The solution was sterilized by autoclaving.

List of Solutions

Trace Element Solution

The composition of trace element solution is shown in Table A2.

Table A2. The composition of trace element solution

Composition	Mg/L
HCl (25% v/v)	1 ml
$ZnCl_2$	70
$MnCl_2.\ 4H_2O$	100
H_3BO_3	60
$CoCl_2.\ 6H_2O$	200
$CuCl_2.\ 2H_2O$	20
$NiCl_2.\ 6H_2O$	20
$NaMoO_4.\ 2H_2O$	40

The ingredients of the solution are dissolved in 1000 ml of distilled water and sterilized by autoclaving.

Fe-citrate solution

0.1 g Fe citrate was dissolved in 100 ml distilled water and sterilized by autoclaving.

Vitamin solution

The composition of vitamin solution is given in Table A3.

Table A3. The composition of vitamin solution

Vitamins	**Mg/L**
Thiamine	500
Niacin	500
Biotin	15

Solutions and Buffers

TE Buffer

10 mM Tris

1 mM EDTA

pH is adjusted to 8.0

TAE Buffer

40 mM Tris-EDTA

1 mM EDTA

pH is adjusted to 8 with acetate

Wash Buffer (for DNA extraction from gel)

15 ml of concentrated solution of Tris, NaCl, and EDTA were dissolved in 285 ml of 95% EtOH

Binding Solution (for DNA Extraction from Gel)

6M of Sodium Iodide

Protoplasting buffer (for RNA Isol. From Gram-Negative Bacteria)

15 mM Tris-Cl

0.45 M Sucrose

8 mM EDTA

pH is adjusted to 8.0

Gram-negative lysing buffer (for RNA Isol. From Gram-Negative Bacteria)

10 mM Tris-Cl

10 mM NaCl

10 mM sodium citrate

1.5 % (w/v) SDS

pH is adjusted to 8.0

Saturated NaCl (for RNA Isol. From Gram-Negative Bacteria)

40 g NaCl in 100ml DEPC-treated H2O

Loading Buffer

0.25% Bromophenol Blue

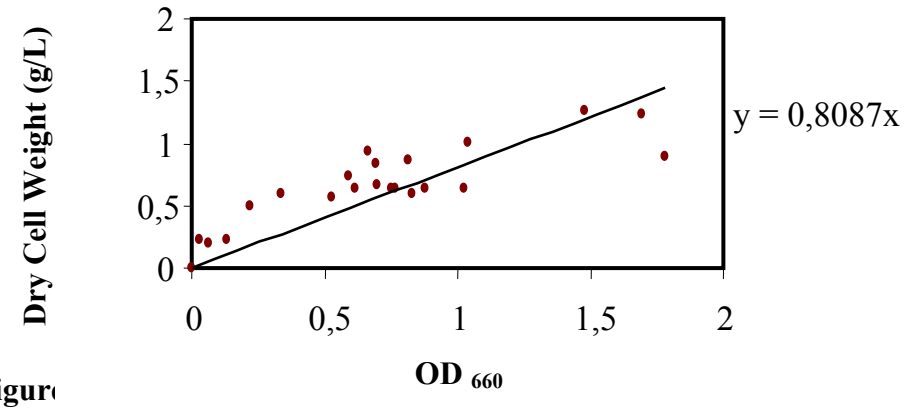
0.25% Xylene cyanol

50% Glycerol

1 mM EDTA

APPENDIX B

DRY CELL WEIGHT vs OD CALIBRATION CURVES



Figur

EXPERIMENTAL DATA

Time	ctrl	pH	No Ni	pH	Ni 1/10X	pH	Ni 10X	pH	Ni 100X	pH
0	0.232	7.17	0.248	7.11	0.224	7.07	0.232	7.19	0.230	7.12
6	0.412	7.40	0.392	7.44	0.394	7.36	0.396	7.46	0.360	7.40
21	1.250	8.52	1.500	8.62	1.520	8.59	1.472	8.71	1.200	8.53
30	1.650	8.40	1.890	8.51	1.800	8.44	1.840	8.55	1.480	8.30
50	2.432	8.80	2.272	8.50	2.300	8.25	2.350	8.86	1.920	8.17
94	2.008	7.80	2.088	7.86	1.988	7.90	1.932	7.90	2.052	7.73
117	1.844	7.66	1.982	7.84	1.942	7.83	1.836	7.72	1.642	7.72

Time	ctrl	pH	No Fe	pH	Fe 1/10X	pH	Fe 10X	pH	Fe 50X	pH
0	0.140	7.01	0.064	7.00	0.130	6.91	0.140	6.97	0.208	7.16
6	1.550	8.20	0.180	7.05	1.460	8.10	1.200	7.92	0.960	7.70
21	2.074	7.98	0.840	7.32	2.110	8.15	1.340	7.57	1.070	7.50
30	1.668	7.94	1.400	8.71	1.624	7.85	1.230	7.56	1.026	7.25
50	1.370	7.70	1.420	8.78	1.320	7.70	1.020	7.40	0.850	7.25
94	1.224	7.30	1.300	7.93	1.208	7.50	0.914	7.40	0.680	7.27
117	0.834	7.78	0.960	7.69	0.960	7.52	0.800	7.59	-	-

Sequence Analysis Results

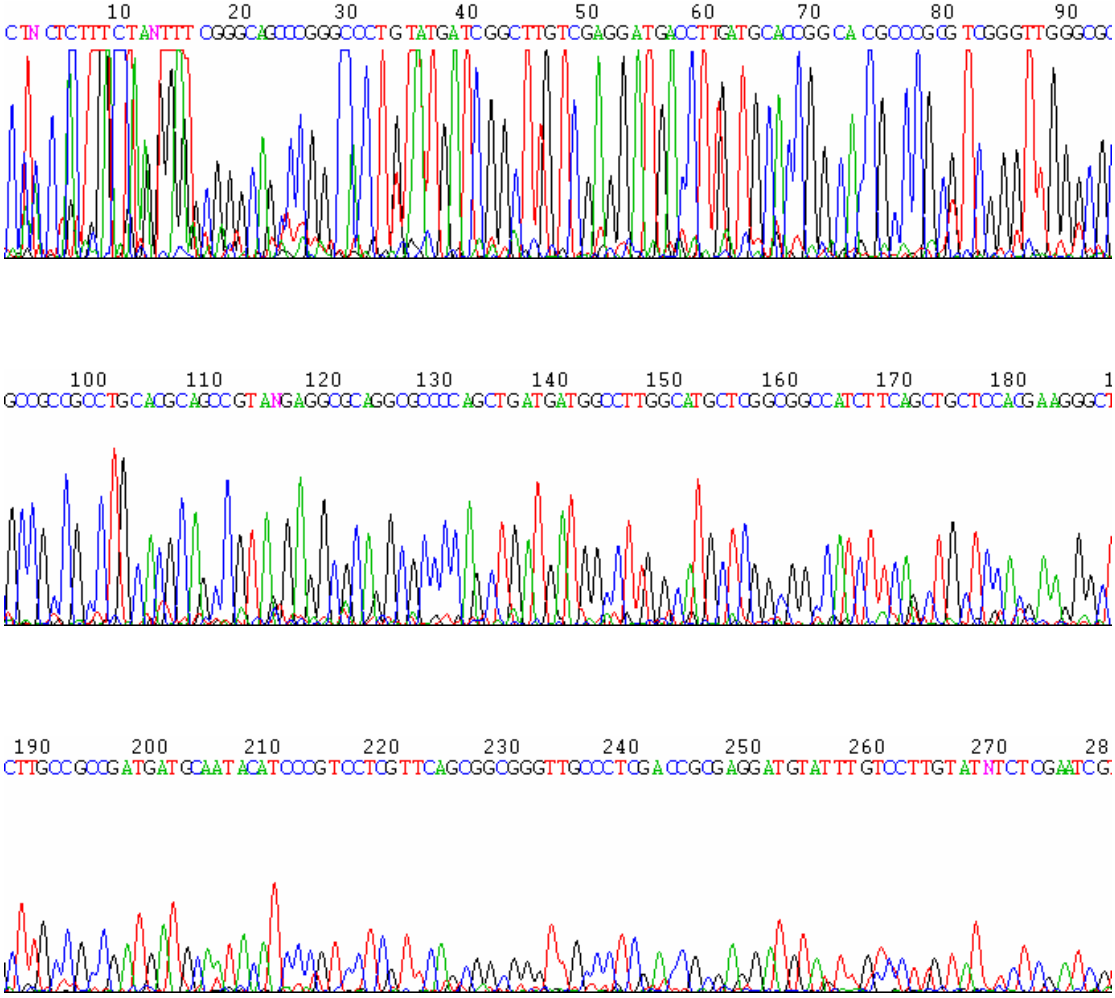


Figure B.2 Sequence analysis results of *R.sphaeroides* O.U.001 *HupS* gene Right Primer

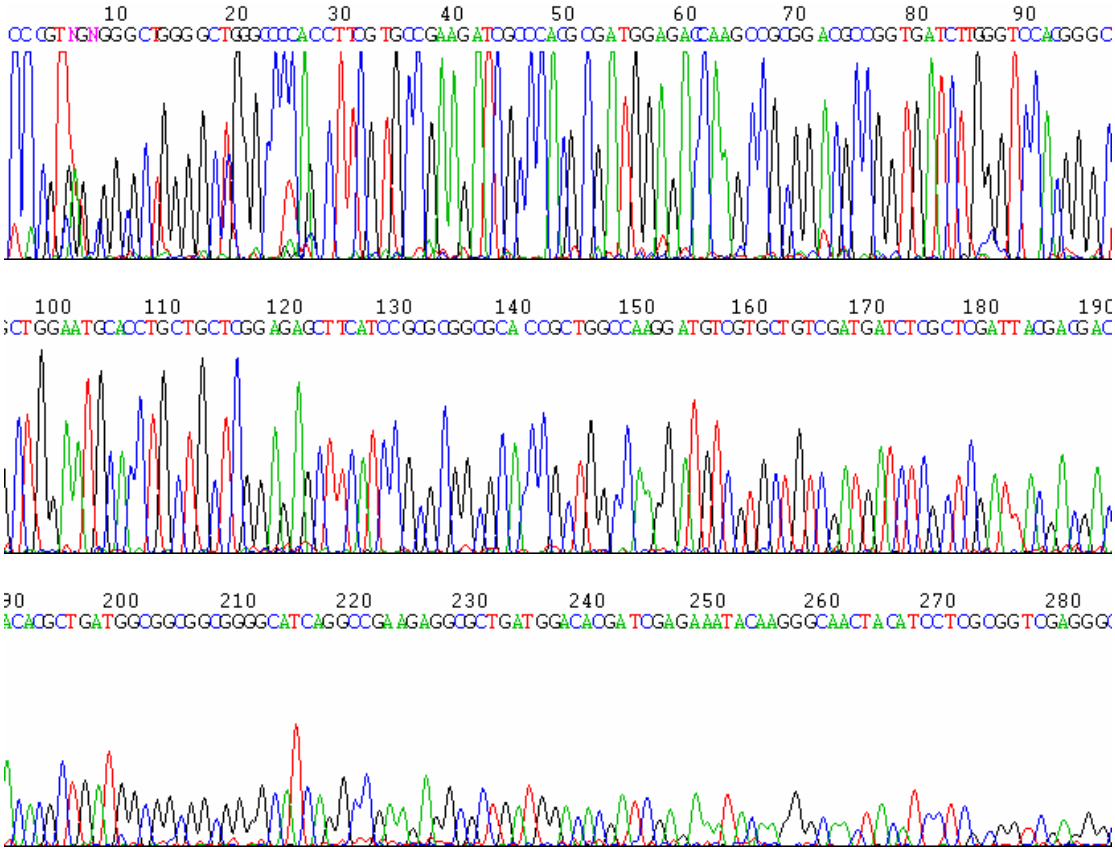


Figure B.3 Sequence analysis results of *R.sphaeroides* O.U.001 *HupS* gene Left Primer

List of Chemicals and Their Suppliers

Chemical	Supplier
KH2PO4, NaCl, MgSO4.7H2O	MERCK
NH4Cl	MERCK
CaCl2.2H2O	MERCK
Malic Acid	MERCK
Glycerol	BIRKA
Sodium Glutamate	MERCK
Yeast Extract	OXOID
Fe-citrate	MERCK
HCl	MERCK
Thiamine, Biotin, Niacin	SIGMA
NaOH	MERCK
ZnCl2, MnCl2.4H2O	MERCK
H3BO3	MERCK
CoCl2.6H2O	MERCK
CuCl2.2H2O	MERCK
NiCl2.6H2O	MERCK
NaMoO4.2H2O	MERCK
Agarose	SIGMA
Chlorophorm	MERCK
EDTA	SIGMA
Ethanol	TEKEL
Isoamyl Alcohol, Isopropanol	MERCK
NaCl	MERCK
Phenol	MERCK
SDS	MERCK
Sucrose	SIGMA
Lysozyme	SIGMA
Taq DNA Polymerase	MBI FERMENTAS
100 bp DNA Ladder Plus	MBI FERMENTAS

DNA Extraction Kit	MBI FERMENTAS
RNA Isolation Kit	QIAGEN